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Growth and metastasis of breast cancer is directly dependent on neovascularization. By understanding the mechanisms that control the neovascular response, it may be possible to design therapeutic strategies to selectively prevent or halt pathological growth of vessels and consequently restrain the progression of cancer cells. Despite its general biological significance and pathological relevance, relatively little is known about inhibitors of blood vessel formation. Thrombospondin-1 (TSP1) has recently been identified as a negative regulator of angiogenesis. Its relevance for the suppression of vascular growth in tumors has yet to be investigated.

The present proposal was designed to address the role of TSP1 in the neovascularization of mammary tumors. Initially, kinetics of vascular development will be examined in mammary tumors of TSP1-deficient mice and in tumors of control animals. A second set of experiments will focus on the effect of TSP1 in normal and tumor-derived endothelial cells at the cellular and at the molecular level. Specifically, we will investigate the proliferation, invasion, and chemotaxis of normal and tumor-derived endothelial cells in a model of angiogenesis *in vitro*. A final facet of this proposal is directed to investigate the modulation of TSP1 receptors as both populations of cells organize into cords and tubes *in vitro* and to identify the receptor(s) responsible for the generation of signals ultimately responsible for the regulation of endothelial cell behavior in breast cancer.

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TABLE OF CONTENTS

INTRODUCTION

1. Abstract of the grant proposal

Growth and metastasis of breast cancer is directly dependent on neovascularization. By understanding the mechanisms that control the neovascular response, it may be possible to design therapeutic strategies to selectively prevent or halt pathological growth of vessels and consequently restrain the progression of cancer cells. Despite its general biological significance and pathological relevance, relatively little is known about inhibitors of blood vessel formation. Thrombospondin-1 (TSP1), a glycoprotein originally described as a major component of platelet α-granules, has recently been identified as a negative regulator of angiogenesis. Its relevance for the suppression of vascular growth in tumors has yet to be investigated.

The present proposal was designed to address the role of TSP1 in the neovascularization of mammary tumors. Initially, kinetics of vascular development will be examined in mammary tumors of TSP1-deficient mice and in tumors of control animals. A second set of experiments will focus on the effect of TSP1 in normal and tumor-derived endothelial cells at the cellular and at the molecular level. Specifically, we will investigate the proliferation, invasion, and chemotaxis of normal and tumor-derived endothelial cells in a model of angiogenesis *in vitro*. A final facet of this proposal is directed to investigate the modulation of TSP1 receptors as both populations of cells organize into cords and tubes *in vitro* and to identify the receptor(s) responsible for the generation of signals ultimately responsible for the regulation of endothelial cell behavior in breast cancer.

2. Relevance of the present work to Breast Cancer

Partial reduction or suppression of tumorigenicity can be accomplished at multiple levels: by direct cell cycle regulation, targeted cellular ablation, control of signal transduction, and/or inhibition of angiogenesis (reviewed in refs. 1-6). Several investigators have implicated tumor suppressor genes in cell cycle regulation or signal transduction pathways and considerable effort is being made to identify the critical points in cell transformation that might be sensitive to pharmacological control. A parallel line of investigation has focused on understanding the **regulation of vascular growth in cancer.** It is recognized that an increase in the vascular supply plays a central role in tumor progression and metastasis (5-9). In fact in breast cancer, angiogenesis has been acknowledged as a significant indicator of tumor progression that is independent of axillary lymph node status (9-11). Although of recognized relevance, therapeutic approaches have generally excluded treatment of breast cancer by target ablation of neovascular growth; mostly because to date, angiogenic inhibitors have proven either too generally toxic or not selective to particular vascular beds. This proposal offers a new perspective into the concept of vascular inhibitors by focusing our attention on a natural angiogenic inhibitor present in normal mammary glands: the glycoprotein thrombospondin-1 (TSP1). According to our preliminary data, TSP1 seems to be suppressed during pathological neovascularization of breast tumors, therefore it is our premise that an exogenous supply of TSP1 should be effective and non-toxic. In addition, TSP1 seems to be specific to steroid-dependent tissues, which could potentially offer selectivity in the inhibition of mammary vessels.

3. Backgound and previous work done by the applicant

TSP1 has been identified as an inhibitor of angiogenesis both *in vivo* and *in vitro* (12-14). Interestingly, TSP1 has also been acknowledged as a tumor-suppressor gene in human-hamster cell hybrids and has been implicated in the inhibition of the tumorigenic ability of MCF-7 breast cancer cells (15). In addition, we recently found that TSP1 mRNA is regulated by steroids at the transcriptional level, an interesting finding considering the implication of steroids in the

development of certain mammary tumors (16) Examination of the vasculature of human mammary glands reveals consistent expression of TSP in the capillaries of normal tissue; its expression in tumors, however is not as steady. TSP1 was indeed rarely present in capillaries of the human tumors examined. Although the casual correlation between lack of TSP1 and rapid growth of tumors is attractive, whether its absence is associated with the rampant growth and capillary progression of those tumors and whether administration of TSP1 could reverse the abnormal growth of capillaries in mammary tumors remains to be tested.

Thrombospondin in the mammary gland and other steroid-dependent tissues.

High levels of TSP1 have been reported in milk, other breast secretions, and in some types of mammary cystic fluids (17). With the exception of bone and circulating platelets, the mammary gland represents one of the few adult tissues with high concentrations of TSP. Interestingly, a study on the kinetics of TSP in human milk has shown temporal variations which could be due to hormonal regulation. TSP was detected in the initial "aqueous phase" of milk secretion, its levels subsequently fell during the transition to mature milk (18). We have studied the expression of TSP1 in 32 hysterectomy cases. Our results showed stage-dependent regulation of this gene in the human endometrium (Table 1 and appendix - manuscript). Specifically we identified TSP1 protein in capillaries of the functional endometrium and this expression correlated with the end of the endometrial cycle (secretory phase). Tissues from the early proliferative phase showed no immunoreactivity. Identification of vessels with an anti-CD 34 antibody in serial sections demonstrated that only a subset of capillaries was reactive with anti-TSP-1 antibodies. Moreover, immunostaining with PCNA (proliferating cell nuclear antigen) IgG indicated that the presence of TSP-1 protein did not, in all instances, correlate with proliferating endothelial cells. Since neovascularization is also regulated by a series of inhibitory signals, we propose that TSP1 is required at later stages of the endometrial cycle to inhibit vessel formation or to stabilize newly-formed capillaries. We have also performed *in situ* hybridization on similar sections to localize TSP1 transcripts. Abundant expression of TSP1 mRNA was identified in the secretory phase, in contrast to the low levels detected in the proliferative phase. TSP1 mRNA was observed not only in endothelial cells, but also in stromal cells of the human endometrium. It was interesting that no protein was detected in stromal cells by immunocytochemistry. These observations suggest that, although stromal cells might secrete high levels of TSP1, the protein is accumulated only in the basement membranes of vessels, where it supposedly exerts its anti-angiogenic effect. Therefore, it is our presumption that TSP1 might act in a paracrine or autocrine manner to regulate vessel growth. Indeed, this hypothesis could be extended to other systems, since significant levels of TSP1 protein are also secreted by vascular smooth muscle cells (19). In the secretory phase, the distinction between the stratum functionalis and the stratum basalis with regard to TSP1 mRNA expression was striking. Whereas high levels were observed in the stratum functionalis, only background levels, equivalent to the intensity observed during the proliferative phase, were seen in the stratum basalis. These findings suggested to us that the TSP1 gene might be, at some level, regulated by steroids.

We have examined the regulation of TSP1 mRNA in cultured cells exposed to progesterone. Steady-state levels of TSP1 mRNA were elevated 4.5 fold in human stromal endometrial cells at 6h after treatment with progesterone. This effect was dose-dependent and was mediated at the transcriptional level, as shown by nuclear run-on experiments. In this context, it is interesting that analysis of the mouse and human TSP1 promoters reveal the presence of a consensus sequence (AGTCCT) (20) that has been reported to interact with the glucocorticoid receptor (21).

In the mammary gland, we have detected TSP1 in the subendothelium of blood vessels (these results were submitted as preliminary data in the grant proposal). When sections of breast cancer were examined for the presence of TSP1, we verified that capillaries were positive in some but not in all types of breast tumors.

The possibility that the TSP1 gene might be regulated by steroids has not been carefully explored, although a number of reports suggest, in an indirect manner, that this type of regulation might occur (22-24). As part of another project we have proposed to investigate this issue further

to locate steroid response elements in the TSP1 gene and identify possible trans-acting factors involved in this regulation (NIH - R29 CA65624-01). Within the context of angiogenesis, it is curious that a number of laboratories have reported inhibition of angiogenesis by steroids (25,26). It would be pertinent to evaluate whether the inhibition of blood vessel formation mediated by steroids has any correlation with the secretion of TSP1.

4. Purpose of the present work

This proposal offers a logical progression to the knowledge previously gained on the role of TSP1 in vascular biology and offers a potentially exciting avenue for the identification of a natural inhibitor of capillary growth for the treatment of human breast cancer. The successful completion of this research project will: **1.** determine whether the lack of TSP1 facilitates tumor progression and enhancement of vascular growth; **2.** identify cellular mechanism(s) by which TSP1 inhibits angiogenesis in endothelial cells; **3.** identify the receptor(s) involved in the modulation of endothelial cell behavior and examine the intracellular signaling mechanisms; **4.** determine whether TSP1 could be a selective marker for tumor-associated angiogenesis, and more importantly, **5.** determine whether the regulation of TSP1 gene can provide a natural pathway for the clinical treatment of breast cancer.

BODY

EXPERIMENTAL METHODS

A. Is the lack of TSP1 associated with growth and metastasis of malignant tumors?

Examine the progression of the vascular bed in mammary tumors of TSP1-deficient mice.

Experimental Desing/Methodology:

1. Generate mammary tumors in TSP1-deficient (tsp/tsp$^-$) mice by mating of TSP1 knock-out homozygotes with mice carrying the MMTV c-*neu* transgene

2. Analysis of the vascular bed, as well as rate of capillary extension/mm^2 of neoplastic tissue will be obtained by: a) confocal laser analysis coupled with three-dimensional reconstruction, b) determination of hemoglobin and c) endothelial cell markers. Values obtained from TSP1-deficient and from control *neu* animals will be compared.

Overall growth of the tumors and rate of metastasis will also be directly assessed and correlated with control values. Data from these experiments will concurrently provide important information on the relationship between capillary density and tumor expansion.

Determine whether exogenous TSP1 can revert/rescue the vascular phenotype of induced tumors in TSP1-deficient mice.

Experimental Desing/Methodology:

1. Slow-release pellets of TSP1 protein will be implanted in the mammary fat pads of TSP1-deficient mice carrying the MMTVc-*neu* transgene.

2. Vascular progression in tumors will be determined and compared to control-*neu* mice.

3. In addition, the localization of exogenous TSP1 protein and its half life in tumors will be assessed to gain information on the fate of exogenous TSP1 in mammary tumors.

B. What are the specific effects of TSP1 on endothelial cells engaged in angiogenesis?

Investigate the specific effect(s) of TSP1 on endothelial cells engaged in the angiogenic response.

1. Endothelial cells (EC) from normal mouse mammary gland and from mammary tumors will be isolated and characterized for their proliferation rate, secretory profile, and angiogenic potential.

2. Exogenous TSP1 will be added to EC at confluence or to cells undergoing angiogenesis *in vitro*. These experiments will be performed in both tumor-derived as well as control cells. We will determine the effect of this addition on: a) proliferation; b) migration; c) chemotaxis; and d) expression of extracellular matrix-associated molecules.

Identify the cell surface receptor(s) involved in mediating cellular responses to TSP1.

1. The presence of TSP receptors will be assessed in cultures of confluent EC, as well as in angiogenic cultures, by direct binding assays.

2. Modulation of receptor number will be analyzed after addition of TSP1.

3. Neutralizing experiments with specific anti-TSP receptor antibodies will be performed to determine which of the five recognized receptors mediates an anti-angiogenic response.

RESULTS

1. Proposed task for year 1

Task 1, Examine the progression of the vascular bed in mammary tumors of TSP1-deficient mice, Months 1-24

a. Generate TSP1-deficient mice containing mammary tumors

b. Tumors from experimental and from control animals will be harvested and measured

c. Analyze the frame-work of capillaries in both experimental settings by morphometry

d. Analyze the frame-work of capillaries in both experimental settings using a biochemical strategy.

2. Achievements for year 1

Task 1, as stated will be completed by the end of next year. Extensive time is required for the generation of spontaneous tumors in c-neu mice. In general c-neu mice develop mammary carcinomas 6-8 months after birth.

At this point our achievements are the following:

1- We have successfully cross-mated c-neu mice and TSP-deficient mice.

2- The predicted Mendelian distribution was observed in Southern blot analysis of genomic DNA from the off-spring mice as follows:

- c/neu + and tsp-/-
- c/neu - and tsp-/-
- c/neu + and tsp-/+
- c/neu - and tsp-/+

Our interest centers in the female population that presents the genotype c/neu + and tsp-/-. Therefore, although 15 successful matings have been concluded only three females c/neu -/- and tsp-/- were obtained. To achieve statistical significance, at least 10 females will be necessary. We are now in the process of mating a larger number of animals to complete task 1. Considering our experience in the first year of the proposal, we feel that this number will be obtained within the time originally requested for the execution of this task (end or year 2).

The three females that had the appropriate phenotype were observed daily for the development of mammary tumors. At this time only one has develop mammary tumors (at 7

weeks after birth). We expect that the other two females will also follow in the development of tumors (since they are younger, 4 and 6 weeks respectively).

Currently we are analyzing two tumors carried by the c/neu + and tsp-/- female and tumors from control animals (c-neu+ and tsp +/+). Although the vascular analysis of the tumor is not concluded, the weight of the tumors is significantly different from controls as shown in Table I.

Table I - Comparison of the weight of mammary tumors from c/neu + and tsp-/- females and controls

Mouse identification	Phenotype	tumor weight (in grams)*
57675	c/neu + and tsp-/-	0.72
57675	c/neu + and tsp-/-	0.89
57630	c/neu + and tsp+/+	0.47
57635	c/neu + and tsp+/+	0.59
57665	c/neu + and tsp+/+	0.28

*Time allowed for the development of the tumors was 8 days in all animals.

Although only one animal has been studied, the results indicate that the absence of TSP1 significantly favors the development of mammary tumors.

In upcoming experiments, we will also analyze heterozygous animals (c/neu+ and tsp-/+) and compare their mammary tumors to tumors from c/neu + and tsp-/- females.

Results from the vascular analysis of the first case are expected to be concluded by the end of November. All the vascular analysis take approximately 6 weeks. In the future we will perform the vascular studies in groups of three to four animals (and respective controls). This will maximize efficiency and will prove helpful for direct comparisons among samples.

3- During this year we have also isolated and fully characterized endothelial cells from tsp-/- and from tsp +/+ animals. Specifically, fifteen isolations from independent animals of each phenotype have been performed. These cells will be used for Tasks 3 and 4 and indicated in the grant proposal.

The strains were characterized for the expression of PECAM-1, vWF, and flt-1 protein and mRNA. Cell proliferation studies were also done with VPF/VEGF and with bFGF. Comparisons between tsp-/- and tsp+/+ revealed interesting differences in the proliferation profile and ability to migrate into collagen gels. Results from these experiments are currently being drafted for a manuscript to be submitted for publication.

All the strains were expanded and frozen at early passage number. If time permits, we will soon initiate the experiments that have been proposed for years 3 and 4.

3. Achievements not proposed in the original grant, but related to this project

1. Studies on cleavage fragments of TSP1 protein

Aim 2 of our research grant proposes experiments to suppress the vascularization of mammary tumors in c-neu mice and in c-neu+ / tsp-/- mice. To estimate the concentration of TSP1 necessary in experiments that are forthcoming (in vivo delivery of TSP1) and to have a clear idea of the functional activity of the different batches of TSP1 protein purified in our laboratory, we devised a functional assay. The assay consists on the ability of TSP1 protein to inhibit migration/invasion of endothelial cells into collagen gels containing bFGF. To suppress any endogenous protein, we use endothelial cells derived from tsp-/- mice and serum-free

conditions. This assay has proven extremely useful to "normalize" our preparations of TSP1 and to guide our protein purification strategy to yield a greater percentage of active / functional TSP1.

For the execution of the assay, we have included heat-denatured TSP1, BSA alone, and protease-denatured TSP1. To our surprise, when we utilized thrombin-cleaved TSP1 the ability of the protein to inhibit migration was 10-15 times enhanced. Further, more refined experiments (thrombin-free) revealed that fragments of TSP-1, generated by thrombin proteolysis, were 35-50 times more potent than the intact protein in inhibiting migration of endothelial cells into collagen gels. These data would indicate that proteolysis is required, or at least enhances, the anti-angiogenic effect mediated by TSP1. Therefore, synthetic peptides that correspond to relevant TSP-1 subdomain(s) should mimic the anti-angiogenic effect mediated by TSP1. These results were submitted, as an administrative supplement grant proposal to our existing grant. The proposal and the reviews of this proposal were included in the Appendix.

These results provided new information on the molecular mechanisms by which TSP1 might exert its anti-angiogenic function. We are very excited about this novel findings, because they can solve problems related to delivery and metabolic processing in *in vivo* experiments. We will re-design experiments proposed in Aim 2 to utilize thrombin-derived fragments of TSP1 (although intact TSP1 will be used).

2. Analysis of the expression of TSP1 and vascular density during physiological expansion of the mammary gland

We have design experiments to evaluate the physiological fluctuation of TSP1 in the mammary gland that has been challenged to undergo physiological growth. For these experiments we have harvest mammary glands from pregnant females (day 7, 16 and day 19), lactating females (day 1, 2, and 7), and from regressing mammary glands (1 day, 2 days, and 7 days regressing). From each animal four glands were harvested (pair 3 and pair 4). The glands were prepared accordingly for: mRNA extraction, protein extraction, fixation in paraformaldehyde for in situ hybridization analysis, and fixation in methacarnoys for immunocytochemical analysis.

These experiments will prove important for two reasons:

(1) to determine the physiological fluctuation of endogenous levels of TSP1 protein and mRNA in the murine mammary gland,

(2) to evaluate whether TSP1 is relevant to the decreased vascularity characteristic of regressing glands;

Although these experiments are ongoing, we have concluded the mRNA analysis. Our results indicate that there is a constant base-line (or constitutive) expression of TSP1 in the mammary gland. However, TSP1 mRNA is, in fact, induced 3-fold in mid-pregnancy (day 16) and 4-6 fold during regression. The significance of these results will become more clear after the analysis of the degree of vasculature are concluded.

3. Analysis of 21 human tumors for the expression of TSP1 mRNA.

One of the concerns expressed by the reviewers of this grant proposal was the fact that the experiments were not being performed in humans, and that direct extrapolation of results might prove inadequate. We were aware of this very real issue, nevertheless, the nature of the experiments proposed could only be executed in experimental animals. However, to validate the execution of these experiments, we felt that it was appropriate to complement our studies with analysis of TSP1 expression in a variety of human mammary tumors.

Preliminary data presented in our research proposal showed that TSP1 protein was not detected by immunocytochemistry in the majority of human tumors with high vascularization. In these experiments, an antibody that recognized the amino-terminal end of the protein (the active anti-angiogenic domain) was used. We have now extended this study to 21 additional mammary tumors. Both analysis of protein and mRNA were executed. Our findings indicate that TSP1 mRNA is highly expressed in most tumors observed. When the same samples were examined for the expression of protein a puzzling result was observed: protein was not seen using an antibody to the amino-terminal region of the protein (consistent with our previous results), but was

detected using an antibody to the carboxy-terminal end. Our initial trivial explanation was that the amino-terminal antibody was no longer working, but the control tissues, including lactating mammary glands fixed in parallel with the tumors demonstrated that the antibody was in perfect condition. We are now excited about these results and think that perhaps mutations in the TSP1 gene might have occur in the tumors and as a result the amino-terminal antibody was not detecting the mutated protein. Another contemplated possibility is that there is rapid degradation of the end-terminal end by local proteases. We intend to perform Western analysis and genomic sequencing to address these issues.

CONCLUSIONS

After one year of execution of this project, we feel that major accomplishments have been achieved:

1. Matings of c-neu mice and tsp-/- mice proved possible and yield positive results.
2. Proteolysis of TSP1 is effective in maximizing the anti-angiogenic activity of the protein This finding will help us refined the *in vivo* experiments proposed in years 2 and 3.
3. TSP1 expression is coincident with the physiological suppression of angiogenesis in the mammary gland.
4. There seems to be a high expression of TSP1 mRNA in human mammary tumors, however the protein might have lost its anti-angiogenic properties due to mutations or proteolytic alterations in the amino-terminal end. Research in this area will be of extreme importance to elucidate the role of TSP1 in the control of the vasculature in mammary tumors.

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25. Crum, R., Szabo, S., and Folkman, J. (1985). A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 230, 1375-1378.
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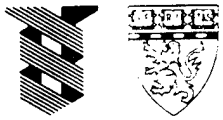
APPENDIX

1. Administrative supplement proposal submitted on 6/10/95 (with reviews)

2. Manuscript in press - *Journal of Clinical Investigation*

Beth Israel Hospital



Harvard Medical School

Beth Israel Hospital
330 Brookline Avenue Boston, MA 02215

epartment of Pathology
esearch North

17) 667-8941
ax (617) 667-3591

June 12, 1995.

National Action Plan on Breast Cancer
PHS Office on Women's Health
Hubert Humphrey Room 730B
200 Independence Ave., SW
Washington, D.C. 20201

Dear Sir or Madam,

Please find enclosed the supplement proposal entitled: "Inhibition of Vascular Growth in Breast Cancer". This is a supplement for the parent proposal: "Suppression of Vascular Growth in Breast Tumors" (DOD/US Army DAMD 17-945-J-4346) in response to the recent announcement: Action Plan on Breast Cancer - Administrative Supplements.

Program Administrator for the Parent Grant:
Ms. Patricia Nelson
U.S. Army Medical Research and
Development Command
Fort Detrick
Frederick, MD. 21702-5014

A copy of the supplement proposal has been sent to Ms. Nelson.

Sincerely yours,

Luisa Iruela-Arispe, Ph.D.
Assistant Professor
Telephone: (617) 667-3614
Fax: (617) 667-3591

Form Approved Through 6/30/94
OMB No. 0925-0001

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

GRANT APPLICATION

Follow instructions carefully. Type in the unshaded areas only. Type density must be 10 c.p.i.

LEAVE BLANK FOR PHS USE ONLY.		
Type	Activity	Number
Review Group		Formerly
Council/Board *(Month, Year)*		Date Received

1. TITLE OF PROJECT (Do not exceed 56 typewriter spaces.)
INHIBITION OF VASCULAR GROWTH IN BREAST CANCER

2a. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT ☐ NO ☒ YES *(If "YES," state number and title)*
Number: NA Title: ACTION PLAN ON BREAST CANCER - ADMINISTRATIVE SUPPLEMENTS

2b. TYPE OF GRANT PROGRAM R03

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME *(Last, first, middle)*
IRUELA-ARISPE, LUISA

3b. DEGREE(S)
PHD

3c. [redacted] PII Redacted

3d. POSITION TITLE
ASSISTANT PROFESSOR

3e. MAILING ADDRESS *(Street, city, state, zip code)*
BETH ISRAEL HOSPITAL
DEPARTMENT OF PATHOLOGY/RN280
330 BROOKLINE AVENUE
BOSTON, MA 02215

3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
PATHOLOGY

3g. MAJOR SUBDIVISION
PATHOLOGY

3h. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: (617) 667-3614
FAX: (617) 667-3591

BITNET/INTERNET ADDRESS liruela@bih.harvard.edu

4. HUMAN SUBJECTS — 4a. X NO ☐ YES — If "Yes," exemption no. or IRB approval date — 4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS — 5a. ☐ NO X YES — If "Yes," IACUC approval date: 092994 — 5b. Animal welfare assurance no.: A3153-01

6. DATES OF ENTIRE PROPOSED PROJECT PERIOD		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR ENTIRE PROPOSED PROJECT PERIOD	
From (MMDDYY)	Through (MMDDYY)	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
093095	092996	22,000	36,740	22,000	36,740

9. PERFORMANCE SITES *(Organizations and addresses)*
BETH ISRAEL HOSPITAL
DEPARTMENT OF PATHOLOGY
330 BROOKLINE AVENUE
BOSTON, MA 02215

10. INVENTIONS AND PATENTS *(Competing continuation application only)*
☐ NO ☐ YES If "YES," ☐ Previously reported ☐ Not previously reported

11. NAME OF APPLICANT ORGANIZATION
BETH ISRAEL HOSPITAL
ADDRESS 330 BROOKLINE AVENUE
BOSTON, MA 02215

12. TYPE OF ORGANIZATION
☐ Public: *Specify* ☐ *Federal* ☐ *State* ☐ *Local*
☒ Private Nonprofit
☐ Forprofit *(General)* ☐ Forprofit *(Small Business)*

13. ENTITY IDENTIFICATION NUMBER: 1042103881A1 — Congressional District: 08

14. BIOMEDICAL RESEARCH SUPPORT GRANT CREDIT
Code: 30 Identification: HOSPITAL

15. NAME OF ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
MR. EUGENE WALLACE
TELEPHONE (617) 667-3675
FAX (617) 667-4483
TITLE VICE PRESIDENT OF FINANCE
ADDRESS BETH ISRAEL HOSPITAL
330 BROOKLINE AVENUE
BOSTON, MA 02215

BITNET/INTERNET ADDRESS

16. NAME OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
MR. MICHAEL LANNER
TELEPHONE (617) 667-4585
FAX (617) 667-4774
TITLE DIRECTOR, RESEARCH ADMINISTRATION
ADDRESS BETH ISRAEL HOSPITAL
330 BROOKLINE AVENUE
BOSTON, MA 02215

BITNET/INTERNET ADDRESS

17. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Willful provision of false information is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).

SIGNATURE OF PERSON NAMED IN 3a. *(In ink. "Per" signature not acceptable.)* — DATE: 6/9/95

18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).

SIGNATURE OF PERSON NAMED IN 16 *(In ink. "Per" signature not acceptable.)* — DATE: 6-12-95

Principal Investigator/Program Director (*Last, first, middle*): IRUELA, ARISPE, Luisa.

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. **DO NOT EXCEED THE SPACE PROVIDED.**

Growth and metastasis of breast cancer is directly dependent on neovascularization. By understanding the mechanisms that control the neovascular response, it may be possible to design therapeutic strategies to selectively prevent or halt pathological growth of vessels and consequently restrain the progression of cancer cells. Despite the potential biological significance and pathological relevance of blood vessel inhibitors, relatively little is known about such agents. Thrombospondin-1 (TSP1), a glycoprotein originally described as a major component of platelet α-granules, has recently been identified as a negative regulator of angiogenesis. However, the efficiency of TSP1 as a tumor suppressor has yet to be investigated.

Although in its early stages (only 7 months after its initiation) our parent project (DOD/US Army DAMD 17-94-J-4346) has made significant progress and has highlighted a novel aspect of tumor cancer progression that might be responsive to intervention by TSP1. As outlined in our parent proposal, we have sucessfully isolated endothelial cells from wild-type and TSP1-deficient mice. Experiments using tsp -/- cells indicated that exogenous TSP1 prevents the invasion of endothelial cells into collagen gels and diminishes the organization of capillary-like structures. Further experiments revealed that fragments of TSP-1, generated by proteolysis, are 35-50 times more potent than the intact protein. These data would indicate that proteolysis is required, or at least enhances, the anti-angiogenic effect mediated by TSP1. Therefore, synthetic peptides that correspond to relevant TSP-1 subdomain(s) should mimic the anti-angiogenic effect mediated by TSP1. If this supplement proposal is granted, we will (**a**) define the specific domains of TSP1 involved in the inhibition of endothelial cell invasion, (**b**) test their efficacy on endothelial cell invasion *in vitro* and (**c**) evaluate their relevance in the suppression of vascular growth of mammary tumors. The successful completion of these experiments will directly determine if TSP1 is a potential candidate for treatment of breast cancer.

PERSONNEL ENGAGED ON PROJECT, INCLUDING CONSULTANTS/COLLABORATORS. *Use continuation pages as needed* to provide the required information in the format shown below on ***all*** individuals participating in the project.

PII Redacted

Name Luisa Iruela-Arispe	Degree(s) Ph.D.	[redacted]
Position Title Assistant Professor	[redacted]	Role on Project Prin. Invest.
Organization Harvard Medical School/Beth Israel Hospital		Department Pathology
Name	Degree(s)	Social Security #
Position Title	D.O.B.	Role on Project
Organization		Department
Name	Degree(s)	Social Security #
Position Title	D.O.B.	Role on Project
Organization		Department
Name	Degree(s)	Social Security #
Position Title	D.O.B.	Role on Project
Organization		Department
Name	Degree(s)	Social Security #
Position Title	D.O.B.	Role on Project
Organization		Department
Name	Degree(s)	Social Security #
Position Title	D.O.B.	Role on Project
Organization		Department
Name	Degree(s)	Social Security #
Position Title	D.O.B.	Role on Project
Organization		Department

RESEARCH GRANT
TABLE OF CONTENTS

*Type density and size must conform to limits provided in Specific Instructions on page 10.

Appendix *(Five collated sets. No page numbering necessary for Appendix)*
Number of publications and manuscripts accepted or submitted for publication *(Not to exceed 10)*: _____
Other items (list):

☐ Check if Appendix is Included

DD

Principal Investigator/Program Director (Last, first, middle): IRUELA-ARISPE, Luisa

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM 09/30/95 THROUGH 09/29/96

PERSONNEL (Applicant Organization Only) NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED (omit cents) SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Luisa Iruela-Arispe, PhD	Principal Investigator	12	20	$67,000	$13,400	$3,484	$16,884
SUBTOTALS →					$13,400	$3,484	$16,884

CONSULTANT COSTS None		$0
EQUIPMENT (Itemize) None		$0
SUPPLIES (Itemize by category) Lab supplies	$5,116	$5,116
TRAVEL None		$0
PATIENT CARE COSTS None	INPATIENT $0 OUTPATIENT $0	
ALTERATIONS AND RENOVATIONS (Itemize by category) None		$0
OTHER EXPENSES (Itemize by category) None		
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD		**$22,000**
CONSORTIUM/CONTRACTUAL COSTS DIRECT COSTS INDIRECT COSTS	TOTAL →	$0
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD	(Item 7a, Face Page) →	**$22,000**

BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
Luisa Iruela-Arispe, Ph.D.	Assistant Professor, Dept. of Pathology, H.M.S. Associate in Pathology, Beth Israel Hospital

EDUCATION *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Univ. Gama Filho, Rio de Janeiro, Brazil	B.S.	1983	Biology
Federal Univ. of Rio de Janeiro, Brazil	M.S.	1986	Histol. & Embryol
Univ. of Washington/Univ. São Paulo	Ph.D.	1989	Cell & Molec. Biol.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

1989-92 Post Doctoral Fellow, Univ. of Washington, Seattle
1992-93 Research Assistant Professor, Dept. of Biological Structures, Univ. of Washington, Seattle.
1994-present Assistant Professor, Dept. of Pathology, Harvard Medical School, Boston.

Honors and other Scientific Recognition — CNP_q Scholarship, Brazil, 1984-1986; CAPES, Brazil, 1986-1987; American Heart Association Fellowship, 1991 - 1993; American Heart Association, Grant-in-Aid, 1993-1996; First-Award, NIH, 1994-1999. Principal Investigator Award, Army Dept. of Defense, 1994-1998; Member: American Society for Cell Biology, North American Vascular Biology Organization, Microbiology Society; American Association for Cancer Research, Society for Developmental Biology, United States and Canadian Academy of Pathology.

Publications:

1. Cotta-Pereira, G. and Iruela-Arispe, M.L. Extracellular matrix: functional significance of oxytalan, elaunin and elastic fibers. *In*: Cells and Tissues: A Three Dimensional Approach by Modern Techniques in Microscopy. Allan R. Liss, Inc. eds. 61-67 (1989).
2. Chow, M., Boyd, C.D. and Iruela-Arispe, M.L., Wrenn, D.S., Mecham, R., and Sage, H. Characterization of elastin protein and mRNA from salmonid fish (Oncorhynchus *kisutch). Comp. Biochem. Physiol.* 93:835-845 (1989).
3. Sage, H., Vernon, R.D., Decker, J. Funk, S. and Iruela-Arispe, M.L. Distribution of the calcium-binding protein SPARC in tissues of embryonic and adult mice. *J. Histochem. Cytochem.* 37:819-829 (1989).
4. Sage, H. and Iruela-Arispe, M.L. Type VIII collagen in murine development. Association with capillary formation *in vitro. In*: Structure, Molecular Biology and Pathology of Collagen. Vol. 580. R. Fleishmajer, B. R. Olsen, and K. Kühn, ed., New York: New York Academy of Sciences, 17-31 (1990).
5. Iruela-Arispe, M.L., Hasselaar, P. and Sage, H. Differential expression of extracellular proteins is correlated with angiogenesis *in vitro. Lab. Invest.* 64:174-186 (1991).
6. Paulus, W., Sage, H., Iruela-Arispe, M.L. and Liszka, U. Selective expression of type VIII collagen in tumors of the human brain. *Brit. J. Cancer* 63:367-371 (1991).
7. Iruela-Arispe, M.L. and Sage, H. Expression of type VIII collagen during morphogenesis of the chicken and mouse heart. *Dev. Biol.* 144:107-118 (1991).
8. Iruela-Arispe, M.L., Bornstein, P. and Sage, H. Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells *in vitro. Proc. Natl. Acad. Sci. USA.* 88:5026-5030 (1991).
9. Iruela-Arispe, M.L., Diglio, C. A. and Sage, E.H. Modulation of extracellular matrix proteins by endothelial cells undergoing angiogenesis *in vitro. Arterioscler. Thromb.* 11:805-815 (1991).
10. Fouser, L., Iruela-Arispe, M.L., Bornstein, P. and Sage, E.H. Transcriptional activity of the α1(I) collagen promoter in endothelial cells is correlated with angiogenesis *in vitro. J. Biol. Chem.* 266:18345-18351 (1991).
11. Raines, E.W., Lane, T., Iruela-Arispe, M.L., Ross, R. and Sage, E.H. The extracellular glycoprotein SPARC interacts with platelet-derived growth factor (PDGF)-AB and BB and inhibits binding of PDGF to its receptor. *Proc. Natl. Acad. Sci.* 89:1281-1285 (1992).

12. Iruela-Arispe, M.L., Chun, L. and Sage, E.H. Structure and biology of type VIII collagen. *Trends Glycosci. Glycotechnol.* 4:188-199 (1992).
13. Vernon RB., Angello, J.C., Iruela-Arispe, M.L., Lane, T.F. and Sage, E.H. Reorganization of basement membrane matrices by cellular traction promotes the formation of cellular networks *in vitro*. *Lab. Invest.* 66:536-547 (1992).
14. Lane, T., Iruela-Arispe, M.L. and Sage, E.H. Regulation of gene expression by SPARC during angiogenesis *in vitro*: changes in fibronectin, thrombospondin-1, and plasminogen activator inhibitor-1. *J. Biol. Chem.* 267:16736-16745 (1992).
15. Sage, E. H., Lane, T.F., Iruela-Arispe, M.L. and Funk, S.E. SPARC: A protein that modulates cell cycle and cell matrix interactions. *Proc. Forth Int'l Conf. Chem. Biol. Mineraliz. Tiss.*, H. Slavkin and P. Price, eds. Elsevier, Amsterdam, 235-242 (1992).
16. Floege, J., Johnson, R.J., Gordon, K., Yoshimura, A., Campbell, C., Iruela-Arispe, M.L., Alpers, C.E. and Couser, W.G. Altered glomerular extracellular matrix synthesis in experimental membranous nephropathy: Studies in two models and cultures glomerular epithelial cells. *Kidney Int.* 42:573-585 (1992).
17. Jäveläinen, H.T., Iruela-Arispe, M.L., Sandell, L.J., Sage, E.H. and Wight, T.N. Modulation of decorin, biglycan and type I collagen synthesis by sprouting bovine aortic endothelial cell cultures. *Exp. Cell Res.* 203:395-401 (1992).
18. Iruela-Arispe, M.L. and Sage, E.H. Endothelial cells exhibiting angiogenesis *in vitro* proliferate in response to TGF-β1. J. *Cell Biochem.* 52:414-430 (1993).
19. Iruela-Arispe, M.L., Liska, D.A., Sage, E.H. and Bornstein, P. Differential expression of thrombospondin 1, 2, and 3 during murine development. *Dev. Dyn.* 197:40-56 (1993).
20. Bassuk, J.A., Iruela-Arispe, M.L., Lane, T.F., Berg, R.A. and Sage, E.H. Molecular analysis of chicken embryo SPARC. *Europ. J. Biochem.* 218:117-127 (1993).
21. Battegay, E.J., Rupp, J., Iruela-Arispe, M.L., Sage, E.H. and Pech, M. PDGF-BB modulates endothelial proliferation and angiogenesis *in vitro* via PDGF β-receptors. *J. Cell Biol.* 125:917-928 (1994).
22. Lane, T.F., Iruela-Arispe, M.L., Johnson, R.S. and Sage, E.H. SPARC is a source of bioactive peptides that stimulate angiogenesis. *J. Cell Biol.* 125:929-943 (1994).
23. Gariano, R.F., Iruela-Arispe, M.L. and Hendrickson, A.E. Vascular development in the primate retina: Comparison of laminar plexus formation in monkey and human. *Invest. Ophtal.Vis. Sci.* 35:3442-3455 (1994).
24. Iruela-Arispe, M.L., Lane, T.F., Redmond, D., Reilly, M., Bolender, R., Kavanagh, T.J., and Sage, E.H. Expression of SPARC during the development of the choriollantoic membrane: protein degradation produces angiogenic peptides. *Mol. Biol. Cell* 6:327-343 (1995).
25. Vernon, R.B., Wight, T.N., Lara, S., Iruela-Arispe, M.L., Angello, J.C., and Sage, E.H. Tesselated networks of endothelial cells that arise spontaneously *in vitro* are associated with traction-generated templates of type I collagen. *Cell Dev. Biol.* 31:120-131 (1995).
26. Seiffert, D., Iruela-Arispe, M.L., Sage, E.H., and Loskutoff, D.J. Distribution of vitronectin mRNA during murine development. *Dev. Dyn.* 203:71-79 (1995).
27. Reed, M.J., Iruela-Arispe, M.L., O'Brien, E.R., Truong, T., LaBell, T., Bornstein, P., and Sage, E.H. Expression of thrombospondins by endothelial cell: injury is correlated with TSP1. *Am J. Pathol.*, in press.

Manuscripts Submitted:

1. Iruela-Arispe, M.L., Porter, P., Bornstein, P. and Sage, E.H. Thrombospondin-1, an inhibitor of angiogenesis is regulated by progesterone in human stromal cells. *J. Clin. Invest.* (1995).
2. Iruela-Arispe, L., Gordon, K., Hugo, C., Duijvestijin, A.M., Claffey, K.P., Reilly, M., Couser, W.G., Alpers, C.E. and Johnson, R.J. Participation of glomerular endothelial cells in the capillary repair of glomerulonephritis. *Am. J. Pathol.* (1995).

Current Support:

		Dates	Role	%Time
1.	NIH 1R29 CA65624-01	04/01/94-03/30/99	PI	50%
2.	DOD/US Army DAMD 17-94-J-4346	10/01/94-09/30/98	PI	20%
3.	AHA 93-1197	07/01/93-06/30/96	PI	5%
4.	MOD 5-FY 93-0936	09/01/93-08/31/95	PI	5%

OTHER SUPPORT

(Use continuation pages if necessary)

FOLLOW INSTRUCTIONS CAREFULLY. Incomplete, inaccurate, or ambiguous information about OTHER SUPPORT could lead to significant delays in the review and/or possible funding of the application. If there are changes in the information after submission, notify the scientific review administrator of the initial review group before the review; if changes occur after the review, notify the appropriate institute.

Other support is defined as all funds or resources, whether Federal, non-Federal, or institutional, available to the principal investigator/program director (and other key personnel named in the application) in direct support of their research endeavors through research or training grants, cooperative agreements, contracts, fellowships, gifts, prizes, and other means. Key personnel are defined as all individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project.

Reporting requirements are: for each of the key personnel, describe (1) all currently ***active*** support and (2) all applications and proposals ***pending*** review or award, whether related to this application or not. If the support is part of a larger project, identify the principal investigator/program director and provide the data for the relevant subproject(s). If an individual has no active or pending support, check "None." Use continuation pages as needed to provide the required information in the ***format*** as shown below.

Name Luisa Iruela-Arispe, PhD Active X Pending ___ None ___

a. Source and identifying no. DOD/US Army DAMD17-94-J-4346 P.I. Luisa Iruela-Arispe, PhD

Title Suppression of Vascular Growth in Breast Tumors

b. Your role on project Principal Investigator % Effort 20

c. Dates and costs of entire project 10/01/94 - 09/30/98 $ 352,704 (DC); $236,312 (IC)

d. Dates and costs of current year 10/01/94 - 09/30/95 $ 88,020 (DC); $ 58,973 (IC)

e. Specific aims of project 1) Examine the progression of the vascular bed in mammary tumors of TSP-1 deficient mice. 2) Determine whether exogenous TSP-1 can revert/rescue the vascular phenotype of induced tumors in TSP-1 deficient mice. 3) Investigate the specific effect of TSP-1 on endothelial cells engaged in the angiogenic response. 4) Identify the cell surface receptor(s) involved in mediating endothelial cell responses to TSP-1.

f. Describe scientific and budgetary overlap None

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

None

OTHER SUPPORT

(Use continuation pages if necessary)

FOLLOW INSTRUCTIONS CAREFULLY. Incomplete, inaccurate, or ambiguous information about OTHER SUPPORT could lead to significant delays in the review and/or possible funding of the application. If there are changes in the information after submission, notify the scientific review administrator of the initial review group before the review; if changes occur after the review, notify the appropriate institute.

Other support is defined as all funds or resources, whether Federal, non-Federal, or institutional, available to the principal investigator/program director (and other key personnel named in the application) in direct support of their research endeavors through research or training grants, cooperative agreements, contracts, fellowships, gifts, prizes, and other means. Key personnel are defined as all individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project.

Reporting requirements are: for each of the key personnel, describe (1) all currently ***active*** support and (2) all applications and proposals ***pending*** review or award, whether related to this application or not. If the support is part of a larger project, identify the principal investigator/program director and provide the data for the relevant subproject(s). If an individual has no active or pending support, check "None." Use continuation pages as needed to provide the required information in the ***format*** as shown below.

Name Luisa Iruela-Arispe, PhD Active X Pending ___ None ___

a. Source and identifying no. NIH 1R29 CA65624-01 P.I. Luisa Iruela-Arispe, PhD

Title Inhibition of the Angiogenic Response

b. Your role on project Principal Investigator % Effort 50

c. Dates and costs of entire project 04/01/94 - 03/30/99 $ 350,000 (DC); $233,576 (IC)

d. Dates and costs of current year 04/01/94 - 03/30/95 $ 45,788 (DC); $ 29,754 (IC)

e. Specific aims of project 1) What is the specific effect of studies on the transcription of TSP1 mRNA in human fibroblasts of the endometrial stoma? 2) Are there steroid-specific regulatory elements (SRE) in the TSP-1 gene? 3) Do proteins that interact with the TSP-1 SREs correspond to previously identified transcription factors? 4) How do the distribution and expression of TSP1 compare in normal and neoplastic mammary gland?

f. Describe scientific and budgetary overlap None

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

None

OTHER SUPPORT

(Use continuation pages if necessary)

FOLLOW INSTRUCTIONS CAREFULLY. Incomplete, inaccurate, or ambiguous information about OTHER SUPPORT could lead to significant delays in the review and/or possible funding of the application. If there are changes in the information after submission, notify the scientific review administrator of the initial review group before the review; if changes occur after the review, notify the appropriate Institute.

Other support is defined as all funds or resources, whether Federal, non-Federal, or institutional, available to the principal investigator/program director (and other key personnel named in the application) in direct support of their research endeavors through research or training grants, cooperative agreements, contracts, fellowships, gifts, prizes, and other means. Key personnel are defined as all individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project.

Reporting requirements are: for each of the key personnel, describe (1) all currently ***active*** support and (2) all applications and proposals ***pending*** review or award, whether related to this application or not. If the support is part of a larger project, identify the principal investigator/program director and provide the data for the relevant subproject(s). If an individual has no active or pending support, check "None." Use continuation pages as needed to provide the required information in the ***format*** as shown below.

Name Luisa Iruela-Arispe, PhD Active X Pending ____ None ____

a. Source and identifying no. American Heart Association #93-1197 P.I. Luisa Iruela-Arispe, PhD

Title Role of TGF-β1 in Angiogenesis

b. Your role on project Principal Investigator % Effort 5

c. Dates and costs of entire project 07/01/93 - 06/30/96 $ 120,000 (DC); $12,000 (IC)

d. Dates and costs of current year 07/01/94 - 06/30/95 $ 40,000 (DC); $ 4,000 (IC)

e. Specific aims of project 1) Effect of TGF-β in the modulation of specific cytokines (PDGF, bFGF, VEGF) known to have angiogenic effects; 2) Determination of TGF-β1 receptor-type and number in subconfluent and angiogenic cultures; 3) Mechanisms of activation of latent TGF-β in angiogenic cultures; 4) Effect of TGF-β on the proliferation of endothelial cells committed to angiogenesis *in vitro*.

f. Describe scientific and budgetary overlap None

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.) None

OTHER SUPPORT

(Use continuation pages if necessary)

FOLLOW INSTRUCTIONS CAREFULLY. Incomplete, inaccurate, or ambiguous information about OTHER SUPPORT could lead to significant delays in the review and/or possible funding of the application. If there are changes in the information after submission, notify the scientific review administrator of the initial review group before the review; if changes occur after the review, notify the appropriate Institute.

Other support is defined as all funds or resources, whether Federal, non-Federal, or institutional, available to the principal investigator/program director (and other key personnel named in the application) in direct support of their research endeavors through research or training grants, cooperative agreements, contracts, fellowships, gifts, prizes, and other means. Key personnel are defined as all individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project.

Reporting requirements are: for each of the key personnel, describe (1) all currently ***active*** support and (2) all applications and proposals ***pending*** review or award, whether related to this application or not. If the support is part of a larger project, identify the principal investigator/program director and provide the data for the relevant subproject(s). If an individual has no active or pending support, check "None." Use continuation pages as needed to provide the required information in the ***format*** as shown below.

Name Luisa Iruela-Arispe, PhD Active X Pending ____ None ____

a. Source and identifying no. March-of-Dimes Basil O'Connor Award; #5-FY93-0936 P.I. Luisa Iruela-Arispe, PhD

Title Neural Crest Defects

b. Your role on project Principal Investigator % Effort 5

c. Dates and costs of entire project 09/01/93 - 08/31/95 $ 54,546 (DC); $5,454 (IC)

d. Dates and costs of current year 09/01/94 - 08/31/95 $ 27,273 (DC); $2,727 (IC)

e. Specific aims of project 1) Compare the composition of the extracellular matrix in wild and cleft-face mutant mice; 2) Define the cellular functions that are affected in mesenchymal cells derived from mutant mice; 3) Rescue of the normal cellular phenotype by the provision of a normal extracellular environment *in vitro*; 4) Rescue of the mutant mice by injection of wild-type cells.

f. Describe scientific and budgetary overlap None

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

This award will finish prior to the initiation of the present proposal.

ABSTRACT OF THE CURRENT PROJECT

Title of the Parent Project: Suppression of Vascular Growth in Breast Tumors
Period of Funding: 10/01/94 - 9/30/98
Identification: DOD/US Army Medical Res. & Dev. #0628

Growth and metastasis of breast cancer is directly dependent on neovascularization. By understanding the mechanisms that control the neovascular response, it may be possible to design therapeutic strategies to selectively prevent or halt pathological growth of vessels and consequently restrain the progression of cancer cells. Despite the potential biological significance and pathological relevance of blood vessel inhibitors, relatively little is known about such agents. Thrombospondin-1 (TSP1), a glycoprotein originally described as a major component of platelet α-granules, has recently been identified as a negative regulator of angiogenesis. However, the efficiency of TSP1 as a tumor suppressor has yet to be investigated.

This research project was designed to address the role of TSP1 in the neovascularization of mammary tumors. We propose to examine the kinetics of vascular development in mammary tumors of TSP1-deficient mice and in tumors of control animals. Additional experiments will focus on the effect of TSP1 in normal and tumor-derived endothelial cells at the cellular and molecular level. Specifically, we will investigate the proliferation, invasion, and chemotaxis of normal and tumor-derived endothelial cells in a model of angiogenesis *in vitro*. A final facet of this proposal is directed to identify the receptor(s) involved in the generation of signals ultimately responsible for the regulation of endothelial cell behavior in breast cancer.

ABSTRACT OF THE SUPPLEMENT PROPOSAL

Although in its early stages (only 7 months after its initiation) our existing project has made significant progress and has highlighted a novel aspect of tumor cancer progression that might be responsive to intervention by TSP1. As outlined in our parent proposal, we have sucessfully isolated endothelial cells from wild-type and TSP1-deficient mice. Experiments using tsp -/- cells indicated that exogenous TSP1 prevents the invasion of endothelial cells into collagen gels and diminishes the organization of capillary-like structures. Further experiments revealed that fragments of TSP-1, generated by proteolysis, are 35-50 times more potent than the intact protein. These data would indicate that proteolysis is required, or at least enhances, the anti-angiogenic effect mediated by TSP1. Therefore, synthetic peptides that correspond to relevant TSP-1 subdomain(s) should mimic the anti-angiogenic effect mediated by TSP1. If this supplement proposal is granted, we will (**a**) define the specific domains of TSP1 involved in the inhibition of endothelial cell invasion, (**b**) test their efficacy on endothelial cell invasion *in vitro* and (**c**) evaluate their relevance in the suppression of vascular growth of mammary tumors. The successful completion of these experiments will directly determine if TSP1 is a potential candidate for treatment of breast cancer.

SPECIFIC AIMS OF THE PROJECT

Specific Aim 1. Determine the domain(s) of TSP1 involved in the inhibition of endothelial cell invasion.

Recombinant polypeptides corresponding to seven specific domains of TSP1 will be generated. We have already subcloned these seven domains of TSP1 into expression vectors. Translation products from those vectors will tested by transient transfection in tsp-/- fibroblasts. Subsequently, baculovirus vectors will be constructed and polypeptides will be purified for *in vitro* and *in vivo* experiments (Aim 2).

Specific Aim 2. Test the efficacy of TSP1 fragments on endothelial cell invasion.

Each polypeptide will be tested initially for effects on endothelial cell invasion into collagen gels. Later, specific subdomains will be tested in mammary tumors by injection of tsp -/- cells stably transfected with the various fragments. The effect of the polypeptides will be evaluated by determination of tumor size and degree of vascularization in the tumor.

BACKGROUND AND SIGNIFICANCE

TSP1 has been identified by us and others as an **inhibitor of angiogenesis** both *in vivo* and *in vitro* (1-3). Interestingly, TSP1 has also been acknowledged as a tumor-suppressor gene in human-hamster cell hybrids and has been implicated in the inhibition of the tumorigenic ability of MCF-7 breast cancer cells (4). In addition, we recently found that TSP1 mRNA is regulated by steroids at the transcriptional level (5), an interesting result considering the implication of steroids in the development of certain mammary tumors. Examination of the vasculature of human mammary glands reveals consistent expression of TSP1 in blood vessels of normal tissue; however TSP1 was rarely present in capillaries of the human tumors we have examined (Iruela-Arispe et al., submitted). Although the casual correlation between lack of TSP1 and rapid growth of tumors is attractive, whether its absence is associated with the rampant growth and capillary progression of those tumors and whether administration of TSP1 could reverse the abnormal growth of capillaries in breast cancer remains to be tested and constitutes one of the objectives of our parent grant proposal.

During the last seven months we have been trying to determine if TSP1 modulates vascularization in the mammary gland with the ultimate objective of utilizing this molecule as a therapeutic agent in tumors of the breast. Our results (Aim 1 of our parent grant) indicate that the rate of capillary progression and vascularization of mammary glands in tsp -/- is at least 2.5 fold higher than in wild-type animals. These data argue that TSP1 plays an active role in the negative regulation of vascularization, at least in the mammary gland.

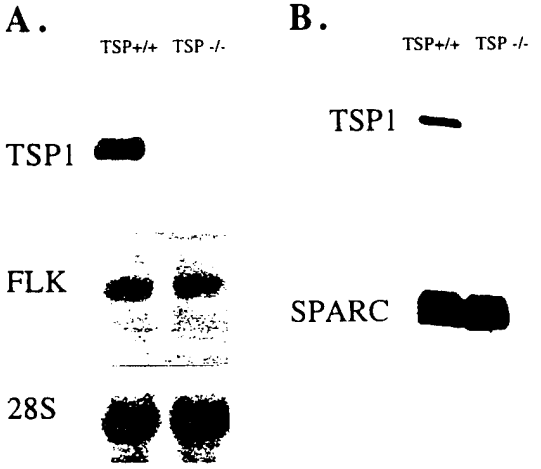
Supporting results also come from our recent evaluation of the effects TSP1 on endothelial cells *in vitro*. Cultured endothelial cells synthesize and secrete large quantities of TSP1, therefore experiments that aim to assess the effect of TSP1 on endothelial cell biology are compromised by endogenous levels of TSP1. Using tsp -/- mice, we have recently isolated and fully characterized endothelial cells that lack TSP1 (Figure 1). We have utilized these cells to determine the effect of TSP1 on endothelial cell behavior. One of the most significant results were observed using *in vitro* angiogenesis assays. Exogenous TSP1 diminished the invasion of endothelial cells into collagen gels (Figure 2), the effect was blocked by TSP1 antibodies and eliminated by heating the protein to 95°C (Figure 2). An interesting finding was that after proteolytic degradation of TSP1 by thrombin, the inhibitory effect was significantly enhanced, however no change was detected when thrombin and carrier alone were used (Figure 2). This is a novel result for TSP1, however proteolytic cleavage of other extracellular matrix proteins has been shown to enhance their properties (6) or even to modify their function (7).

In summary, our results indicate that proteolytic cleavage of TSP1 enhances its anti-angiogenic effect. From these data one could hypothesize that delivery of intact TSP1 might be of some efficacy for the inhibition of angiogenesis, but fragments of TSP1 might provide even greater impact in the suppression of tumor-induced angiogenesis. Furthermore, given the large size and trimeric structure of TSP1, it is also clear that such fragments would be of easier production and delivery than the full size protein. We are now trying to separate the cleavage fragments to obtain information regarding the region of TSP1 responsible for this effect; pilot experiments suggest that the carboxy-terminus might be involved.

A recent report by Weinstat-Saslow and colleagues (8) indicated that overexpression of TSP1 decreased the size of mammary tumors and reduced slightly the number of capillaries. The authors suggested that the decrease in tumor size could be attributed to a partial block in angiogenesis. In light of our recent findings, it is possible that efficient proteolytic cleavage of TSP1 could be an important cofactor for the reduction of blood vessels and to obtain a more dramatic outcome in tumor size. The findings described by Weinstat-Saslow and colleagues provide additional support for this approach and indicate that a more thorough investigation on the effects of TSP1 on mammary tumors, particularly with respect to TSP1 fragments, is warranted. The knowledge gained from our existing project and the reagents now at hand (ie, fibroblasts and endothelial cells from tsp -/- and wild-types animals) place us in an unique position for the execution of these experiments. We believe that the successful completion of the aims described in this research proposal, together with our parent grant, will provide important insight in alternative treatments for breast cancer.

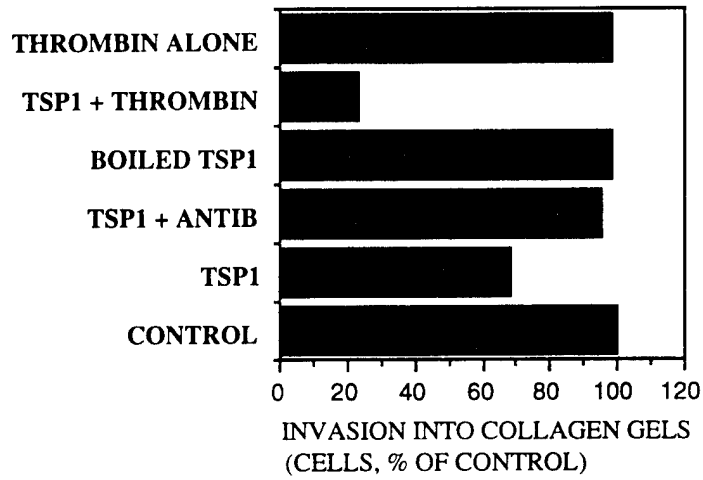
The relevance of blood vessel growth in the progression of mammary tumors has been established (9-11) and several research programs have focused attention on the identification of pharmacological angiogenic inhibitors as means to control cancer progression. Our approach is similar, however, offers the advantage of investigating a natural inhibitor of blood vessel progression that is normally expressed in mammary tissue, but is suppressed during tumorigenic transformation. A more complete understanding of tumor-mediated angiogenesis, in particular the kinetics of inhibitors normally associated with **breast capillaries**, could prove practical in the development of a clinical strategy to control blood vessel growht in breast cancer.

FIGURE 1



A. Northern blot analysis of endothelial cells from tsp+/+ and tsp -/- animals. Probes are indicated. Flk is an endothelial cell marker. 28S cDNA was used to evaluate loading and transfer efficiency.
B. Western blot analysis of conditioned medium from endothelial cells of tsp+/+ and tsp -/- animals. Antibodies were used simultaneously and are indicated. SPARC is a secreted product of endothelial cells and was used as a control.

FIGURE 2



Endothelial cells from tsp-/- mice were plated onto type I collagen gels (30µg/ml) that contained 10ng/ml of bFGF. Cultures were treated with DMEM alone (control); TSP1; TSP1 + antibody; Boiled TSP1; TSP1 previously cleaved by thrombin; and thrombin alone for 2 days. Invasion was determined by direct cell counting. Results were normalized to control.

DIFFERENCES BETWEEN THE PARENT AND THE PRESENT PROPOSAL

It is important to point out the basic differences and complementary points between our original proposal and the present one. The **granted** study proposes to achieve the following objectives: **1.** determine whether the lack of TSP1 facilitates tumor progression and enhancement of vascular growth; **2.** identify cellular mechanism(s) by which TSP1 inhibits angiogenesis in endothelial cells; **3.** identify the receptor(s) involved in the modulation of endothelial cell behavior and examine the intracellular signaling mechanisms; **4.** determine whether TSP1 could be a selective marker for tumor-associated angiogenesis, and more importantly, **5.** determine whether the regulation of TSP1 gene can provide a natural pathway for the clinical treatment of breast cancer.

We have nearly concluded the first objective and have initiated the second one. If granted, the present study will allow us to expand on the last objective and develop research with direct implications to the treatment of breast cancer. We intend to build on the knowledge gained from the results presented and directly test TSP1 polypeptides for their ability to suppress the vascularization of mammary tumors.

The present proposal is a logical complement to the previous grant that can be developed in parallel if resources for personnel and reagents are made available.

RESEARCH PROJECT DESIGN AND METHODS

Specific Aim 1. Determine the domains of TSP1 involved in the inhibition of endothelial cell invasion.

Rationale: Our preliminary experiments have indicated that thrombin cleavage of TSP1 resulted in a potent inhibition of endothelial cell invasion. Identification of the specific bioactive TSP1 domain(s) will be relevant for the generation of synthetic compounds that could mimic the effect obtained with thrombin-cleaved TSP1. Our preliminary studies indicate that the active fragments might be located in the carboxy-terminal end of the protein (where the RGD site is located).

Strategy: We have in hand the complete cDNA for both the human and the mouse TSP1 and have isolated and subcloned seven fragments coding for TSP1 domains (see Figure 3). We will initially test the expression of these domains by transient transfection on tsp-/- cells, and will then generate vectors for expression in baculovirus for recombinant production of polypeptides.

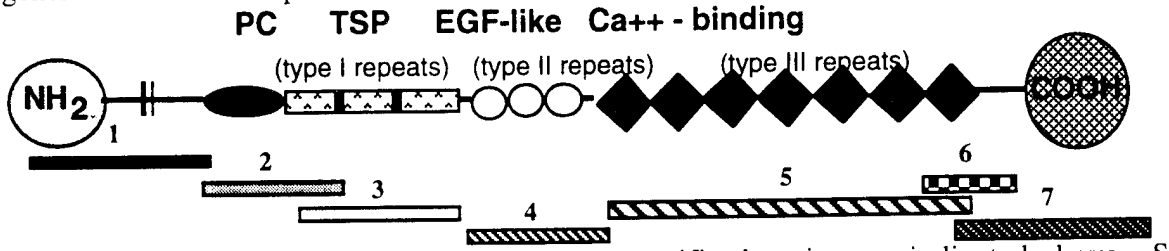


FIGURE 3 - Diagram of the TSP1 protein. Specific domains are indicated above. Seven fragments (indicated) corresponding to each domain have been cloned into expression vectors.

Methodology:

A. Test the expression and stability of the initial constructs by transient transfections: For these assays we will use endothelial cells isolated from tsp-/- mice (Figure 1). Transient transfections will be performed as previously described (12). Two days after transient transfection, we will proceed with RNA extraction to verify the existence of the corresponding mRNA by Northern blot analysis. Concurrently, we will analyze protein synthesis and secretion on Western blots. We have antibodies to the amino-terminal region and to the carboxy-terminal region of TSP1, these antibodies would recognize all, but fragments 4 and 5 (Figure 3), for which we will use polyclonal antibodies.

B. Production of TSP1 fragments in the baculovirus expression system: The TSP1 fragments will be subcloned into pFastBac1 to transform DH10Bac E. coli. After selection of the recombinant Bacmid colonies, we will isolate high molecular weight DNA to transfect insect cells. Finally the recombinant baculovirus particles will be used to infect insect cells and obtain the recombinant fragments. Isolation and purification of recombinant protein will be performed on antibody columns.

Pitfalls and Experimental Problems: At times, the generation of recombinant protein can be difficult to achieve; nevertheless, expression of the entire TSP1 protein and other large TSP1 fragments has been previously accomplished by several investigators, including our collaborator Dr. Jack Lawler (Dept. of Pathology, Brigham and Women's Hosp.). The fragments that we propose to generate are smaller than the ones made in the past, therefore we do not anticipate problems that could not be overcome. However, in the case of a major impediment, we could also test recombinant (larger) TSP1 fragments provided by Dr. Lawler (a letter from Dr. Lawler is appended to the parent proposal).

Specific Aim 2. Test the effects of TSP1 fragments on endothelial cell invasion.

Rationale: We have demonstrated that thrombin-cleaved TSP1 inhibits the invasion of endothelial cells into collagen gels. Verification of this result with purified fragments is required to validate our findings, to localize the important sites, and to prove the feasibility of their use as therapeutic agents in breast cancer.

Strategy: Polypeptides will be tested for their effect on endothelial cell invasion of collagen gels, and later for their effect in mammary tumors. This latest goal can be achieved by two strategies: (a) injection of tumor cells stably transfected with fragments of TSP1 (see Aim 1, Strategy), or (b) introduction of slow release capsules, that contain purified recombinant polypeptides, near sites of tumor growth.

Methodology:

A. In vitro assays: The collagen gel invasion assays have been performed previously by us and constitute some of the preliminary data shown in this proposal (Figure 2). Cells are cultured on a thick gel of type I collagen (Celtrix Corp.) at 30μg/ml containing 10ng/ml of bFGF. Invasion can be detected by direct cell counting of stained nuclei or by staining with ethidum bromide, followed by digestion of collagen, extraction of cells, and measurement of ethidium bromide binding by spectrophotometry.

B. In vivo assays: For these experiments we will use MMTV*c-neu* transgenic mice. In our hands, the *c-neu* mouse has proven a relatively rapid and efficient model for the study of mammary carcinoma. We currently have a colony of these animals for the generation of tsp1-/- /*c-neu* mice (Aim 1, parent grant). In this supplemental grant, we will evaluate the effect of TSP1 fragments by two procedures: **a)** injection of wild-type isogenic, or nude, mice with tsp-/- mammary tumor cells stably transfected with the fragment(s) proven to be effective *in vitro;* **b)** delivery of TSP1 fragments as slow release pellets into tumors of MMTV-*c-neu* mice. This last procedure has been described in our parent grant.

Determination of the effect of TSP1 fragments in mouse mammary tumors will be performed by analysis of several vascular parameters which include: (a) morphometric/confocal analysis and (b) biochemical assays for hemoglobin and lectin *Bandeirae simplicifolia* content in tissue homogenates. These techniques have been described in detail in our parent grant and are currently being utilized for assessment of vascularity in tsp-/- and tsp+/+ mice.

Pitfalls and Experimental Problems: No problems are anticipated for the collagen invasion assays. We are familiar with these experiments and have both endothelial cells derived from tsp-/- and tsp+/+ mice (Figure 2). The generation of cell lines transfected with fragments of TSP1 should also be straight forward, we have performed transfections previously on fibroblasts and on endothelial cells (12) and expression of the entire TSP1 cDNA has been achieved by others (8), therefore this experiment should be feasible. Quantitation of the effect of TSP1 fragments in the vascularization of mammary tumors will be performed by morphometry and confocal microscopy as indicated in our parent grant. We are currently using these techniques and are not anticipating difficulties that cannot be overcome.

DISCUSSION OF THE PROJECT MERITS, PROMISE AND RELEVANCE TO THE PRIORITY AREA AND TO BREAST CANCER

1. Relevance of anti-angiogenesis therapy in breast cancer:

The dependency of tumor growth on vascularization was demonstrated over three decades ago by Dr. Folkman and colleagues. The initial studies demonstrated that tumors maintained in organ culture cease to grow at a density of 1-2mm (13). In analogy, tumors placed in a cornea pocket within 6mm of the iris promoted neovascularization, grew exponentially, and metastasized (14). Over the past thirty years this original hypothesis has gained widespread acceptance and has been established for a variety of tumors (15,16).

The growth and metastasis of breast cancer is no exception to the rule of "vascular-dependency". Tumor-mediated angiogenesis has been identified as a significant and independent prognostic indicator of early stage breast carcinoma and an absolute requirement for expansion and metastatic progression of malignant cells (9-11).

The possibility that tumor progression could be regulated by pharmacological and/or genetic suppression of blood vessel growth has engendered a long-standing interest in the identification of molecules or synthetic compounds that block angiogenesis.

2. Relevance of the present project to breast cancer research:

A number of experiments performed by us and others have established the relevance of TSP1 as a natural inhibitor of angiogenesis (1-3). Furthermore experiments performed by Weinstat-Saslow and colleagues (8) have established the effect of overexpression of TSP1 **directly** in mammary tumors. We have experimental evidence that indicates that a subfragment(s) of TSP1 is 35-50 times more efficient than the intact TSP1, used in that study (8). This proposal will enable us to evaluate the potential of TSP1 fragments as therapeutic agents in mammary carcinomas in mice. We predict that the successful conclusion of these studies will provide sufficient ground to consider TSP1 peptides as strong candidates for clinical trials.

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CHECKLIST

TYPE OF APPLICATION

___ NEW application *(This application is being submitted to the PHS for the first time.)*

___ REVISION of application number: _____
(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

___ COMPETING CONTINUATION of grant number: _____
(This application is to extend a funded grant beyond its current project period.)

X SUPPLEMENT to grant number: D.O.D. #DAMD17-94-J-4346
(This application is for additional funds to supplement a currently funded grant.)

___ CHANGE of principal investigator/program director.
Name of former principal investigator/program director: _____

___ FOREIGN application, city and country of birth and present citizenship of principal investigator/program director. *(This information is required by the U S Department of State.)* _____

1. ASSURANCES/CERTIFICATIONS

The following assurances/certifications are made by checking the appropriate boxes and are verified by the signature of the OFFICIAL SIGNING FOR APPLICANT ORGANIZATION on the FACE PAGE of the application. Descriptions of individual assurances/certifications begin on page 24 of Specific Instructions

a. Human Subjects *(Complete Item 4 on the Face Page)* NONE
☐ Full IRB Review ☐ Expedited Review

b. Vertebrate Animals *(Complete Item 5 on the Face Page)*

c. Inventions and Patents *(Competing Continuation Application Only—Complete Item 10 on the Face Page)*

d. Debarment and Suspension ☒ No ☐ Yes *(Attach explanation)*

e. Drug-Free Workplace *(Applicable only to new or revised applications being submitted to the PHS for the first proposed project period, Type 1)*
☒ Yes ☐ No *(Attach explanation)*

f. Lobbying

With Federal appropriated funds ☒ No

With other than Federal appropriated funds ☒ No ☐ Yes
(If "Yes," see page 29 and attach Standard Form LLL, "Disclosure of Lobbying Activities," to the application behind the second page of the Checklist.)

g. Delinquent Federal Debt ☒ No ☐ Yes *(Attach explanation)*

h. Misconduct in Science (Form PHS 6315) ☒ Filed ☐ Not Filed
If filed, date of Initial Assurance or latest Annual Report 12/28/89

i. Civil Rights Form HHS 441	j. Handicapped Individuals Form HHS 641	k. Sex Discrimination Form HHS 639-A	l. Age Discrimination Form HHS 680
☒ Filed	☒ Filed	☒ Filed	☒ Filed
☐ Not Filed	☐ Not Filed	☐ Not Filed	☐ Not Filed

CHECKLIST (Continued)

2. **PROGRAM INCOME** (See instructions, page 32)

All applications must indicate (Yes or No) whether program income is anticipated during the period(s) for which grant support is requested.

X No ___ Yes If "Yes," use the format below to reflect the amount and source(s) of anticipated program income.

Budget Period	Anticipated Amount	Source(s)

3. **INDIRECT COSTS**

Indicate the applicant organization's most recent indirect cost rate established with the appropriate DHHS Regional Office, or, in the case of forprofit organizations, the rate established with the appropriate PHS Agency Cost Advisory Office. If the applicant organization is in the process of initially developing or renegotiating a rate, or has established a rate with another Federal agency, it should, immediately upon notification that an award will be made, develop a tentative indirect cost rate proposal. This is to be based on its most recently completed fiscal year in accordance with the principles set forth in the pertinent *DHHS Guide for Establishing Indirect Cost Rates*, and submitted to the appropriate DHHS Regional Office or PHS Agency Cost Advisory Office. Indirect costs will **not** be paid on foreign grants, construction grants, grants to Federal organizations, and grants to individuals, and usually not on conference grants. Follow any additional instructions provided for Research Career Development Awards, Institutional National Research Service Awards, and the specialized grant applications listed on page 6.

X DHHS Agreement dated 09/27/93 ☐ No Indirect Costs Requested.

___ DHHS Agreement being negotiated with _____ Regional Office.

___ No DHHS Agreement, but rate established with _____ Date _____

CALCULATION*

(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as CONFIDENTIAL information. Supplying the following information on indirect costs is OPTIONAL for forprofit organizations.)

a Initial budget period
Amount of base $ 22,000 x Rate applied 67 % = Indirect costs (1) $ 14,740

b Entire proposed project period
Amount of base $ 22,000 x Rate applied 67 % = Indirect costs (2) $ 14,740

(1) Add to total direct costs from form page 4 and enter new total on FACE PAGE, Item 7b.
(2) Add to total direct costs from form page 5 and enter new total on FACE PAGE, Item 8b.

*Check appropriate box(es)

___ Salary and wages base X Modified total direct costs base ☐ Other base (Explain below)

___ Off site, other special rate, or more than one rate involved (Explain below)

Explanation (*Attach separate sheet, if necessary.*)

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Washington DC 20201

September 5, 1995

Luisa Iruela-Arispe, Ph.D.
Beth Israel Hospital
Department od Pathology / RN280
330 Brookline Avenue
Boston, MA 02215
REFERENCE: OWH #00111

Dear Applicant:

The National Action Plan on Breast Cancer (NAPBC), a public/private partnership that is working to eradicate breast cancer as a threat to the lives of women, recently reviewed grant applications to meet goals outlined in the Plan. The implementation of the Plan's activities is administered by the U.S. Public Health Service's (PHS) Office on Women's Health. Enclosed is a copy of the Administrative Supplement Evaluation based on comments from the *ad hoc* Review Panel that recently evaluated your grant application to the NAPBC. On the Administrative Supplement Evaluation you will find a priority score which is the average of the scores given individually and privately by each voting member of the Review Panel. The priority score scale ranges from 100 to 500, with 100 representing the highest technical merit.

In deciding which administrative supplement applications to fund, programmatic needs and NAPBC priority area balance will be considered in addition to the technical merit score to achieve the goals of the NAPBC. Therefore, no conclusions should be made concerning the probability of funding based solely on priority score. Decisions about which applications will be awarded are not yet final.

Please note that if your application is funded, the Review Panel's recommended direct costs are subject to the National Cancer Institute's (NCI) grants management staff evaluation, which may result in funding at less than the recommended level. Because the timeframe for awarding these applications is very short, successful applicants may not receive notification of their awards until early October. All applicants, whether or not they receive an award, will be notified in writing of the outcome by mid-October. If you have questions concerning the Administrative Supplement Evaluation of your application, you may call the PHS Office on Women's Health at (202) 401-9587.

Sincerely,

Susan J. Blumenthal, M.D., M.P.A.
Co-Chair, NAPBC
Deputy Assistant Secretary for Health
(Woman's Health)
Assistant Surgeon General

Enclosure

OFFICE ON WOMEN'S HEALTH
NATIONAL ACTION PLAN ON BREAST CANCER
ADMINISTRATIVE SUPPLEMENT EVALUATION

Application Number: 00111 **Score**: 121

Principal Investigator: Luisa Iruela-Arispe, Ph.D.

Institution: Beth Israel Hospital

Title: Inhibition of Vascular Growth in Breast Cancer

Brief Description of Project: Growth and metastasis of breast cancer is directly dependent on neovascularization. By understanding the mechanisms that control the neovascular response, it may be possible to design therapeutic strategies to selectively prevent or halt pathological growth of vessels and consequently restrain the progression of cancer cells. Despite the potential biological significance and pathological relevance of blood vessel inhibitors, relatively little is known about such agents. Thrombospondin-1 (TSP-1), a glycoprotein originally described as a major component of platelet alpha-granules, has recently been identified as a negative-regulator of angiogenesis. However, the efficiency of TSP-1 as a tumor suppressor has yet to be investigated. This laboratory has successfully isolated endothelial cells from wild-type and TSP-1-deficient mice. Experiments using tsp -/- cells indicated that exogenous TSP-1 prevents the invasion of endothelial cells into collagen gels and diminishes the organization of capillary-like structures. Further experiments revealed that fragments of TSP-1, generated by proteolysis, are 35-to-50 times more potent than the intact protein. These data would indicate that proteolysis is required, or at least enhances, the anti-angiogenic effect mediated by TSP-1. Thus, synthetic peptides that correspond to relevant TSP-1 subdomain(s) should mimic the anti-angiogenic effect mediated by TSP-1. This supplement proposes to: 1) define the specific domain(s) of TSP-1 involved in the inhibition of endothelial cell invasion, 2) test their efficacy on endothelial cell invasion in vitro, and 3) evaluate their relevance in the suppression of vascular growth of mammary tumors.

Overall Critique: This is an extremely lucid and well written application that proposes to examine the topical and very significant area of tumor neovascularization. Supplementing already promising initial findings of studies performed in the funded parent grant on angiogenesis suggest that Thrombospondin-1 (TSP-1) -/- murine endothelial cells are unable to penetrate collagen gels and that fragments of TSP-1 are more potent than the intact protein. This application clearly delineates the rationale for the proposed experiments, the research design and methods, and the relevance to breast cancer. There is a very clear distinction between the parent grant and the supplemental application. The reviewers were unanimous in their high level of enthusiasm for this application.

Strengths: The strong data, generated by the parent grant, are significant. The application clearly demonstrates the ability of the investigators to perform the proposed experiments and obtain significant data that would be relevant to clinical breast cancer treatment. In addition, the expertise of the Principal Investigator, coupled with the availability of most materials and technologies necessary for the performance of the proposed experiments, are major strengths of the application. The proposed work is feasible to complete during the proposed funding period.

Weaknesses: Although it is not established that TSP-1 is relevant to mammary cancer, the literature does support the hypothesis that TSP-1 is a natural inhibitor of angiogenesis and that TSP-1 is over-expressed in mammary tumors (although it is unclear whether in tumor cells or in tumor endothelial cells). Even though the data presented by the application are not able to demonstrate that TSP will be relevant to breast cancer, it is none the less highly likely that the work will have practical clinical significance. The use of slow release pellets into the tumors of MMTV-c-neu mice has been criticized by the reviewers of the parent grant, however, the Principal Investigator feels confident that consistent results can be obtained with this methodology.

Budget: The budget is reasonable and justified, and is recommended as requested.

Manuscript # 95-1538R
Submission of Revised Manuscript

THROMBOSPONDIN-1, AN INHIBITOR OF ANGIOGENESIS, IS REGULATED BY PROGESTERONE IN THE HUMAN ENDOMETRIUM

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Running Title: Thrombospondin-1 in the human endometrium

[1]*Abbreviations used in this manuscript:* BAE, bovine aortic endothelial cells; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; FITC, fluorescein isothyocyanate; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; RT-PCR, reverse transcriptase-polymerase chain reaction; TSP1, thrombospondin 1.

Abstract

Thrombospondin-1 (TSP1), a multifunctional extracellular matrix glycoprotein, has been shown to suppress the angiogenic response in vivo and in vitro. We hypothesized that TSP1 might play a role in the inhibition of capillary morphogenesis during the endometrial cycle and examined its expression in 46 human endometrial specimens. Our results show that the expression of TSP1 in the endometrium is (a) cycle-dependent, (b) associated with periods of low capillary growth, and (c) regulated by progesterone. TSP1 protein was identified in the basement membrane of capillaries of the functional endometrium during the secretory phase. Abundant expression of TSP1 mRNA in the secretory phase was also detected by in situ hybridization, in contrast to the low levels seen in the proliferative phase. These findings were confirmed by Northern analysis of proliferative and secretory endometrium. Transcripts for TSP1 were observed predominantly in stromal cells, but signal was also detected in some endothelial and smooth muscle cells. Since the proliferation of endometrial tissue is regulated by steroid hormones, we tested the effects of estrogen and progesterone on TSP1 expression by stromal cells isolated from human endometrium. We found that levels of TSP1 mRNA and protein were increased after incubation with progesterone. Maximal stimulation of mRNA was observed after 8h of treatment with 10-50 μM progesterone, and the effect was suppressed by the progesterone antagonist RU-486. Induction by progesterone was cell-specific and equivalent to the stimulation mediated by PDGF. Finally, the levels of TSP1 present in progesterone-stimulated cultures were sufficient to inhibit the migration of endothelial cells in vitro; this effect was nullified by anti-TSP antibodies. We therefore propose that the production of TSP1 at later stages of the endometrial cycle is linked to the inhibition of vessel formation and that TSP1 expression is progesterone-dependent in this tissue.

Keywords: Blood vessels, extracellular matrix, endometrial cycle, endothelial cells, steroids.

Introduction

Under the stimulatory influence of certain cytokines, endothelial cells convert from a quiescent state to an invasive and proliferative phenotype; this change results in the budding of new capillaries, a process known as angiogenesis. In the normal adult, the expansion of capillary beds is mostly restricted to repair of injury; however, the human endometrium is an exception. Endometrial cycling demands the recurrent growth of new capillaries, since the need for additional vasculature is constantly imposed by the cyclic renewal of the endometrial mucosa. The angiogenic response in the endometrium appears to be tightly controlled, in that accelerated growth is followed by an equally rapid inhibition of growth. Therefore, this tissue provides an interesting model for the study of natural inhibitors of blood vessel morphogenesis in humans.

Thrombospondin-1 (TSP1)[1], a high molecular weight trimeric glycoprotein originally identified in platelet α-granules, was later described as a major secretory product of vascular smooth muscle cells and endothelial cells (1-3). In endothelial cells, TSP1 has been shown to inhibit proliferation (4), disrupt focal adhesions (5), diminish cell spreading (6), and inhibit angiogenesis (7-12). TSP1 suppressed the neovascular response mediated by bFGF in the rat cornea (8), and inhibited capillary formation in vitro (9-10). The region responsible for the antiangiogenic effect has been mapped to the procollagen homology sequence and the type I repeats (11). The specific mechanism that results in angiogenic suppression is not understood and is further complicated by the interaction of TSP1 with a variety of extracellular macromolecules and growth factors. For example, in the presence of type I collagen, TSP1 appears to facilitate rather than inhibit neovessel formation in organ culture (13), and TSP1 binds and activates TGF-ß (14,15), a recognized angiogenic growth factor (16,17). Therefore, the nature of the extracellular environment and/or the presence of TSP1 as a bound or soluble protein, might determine the ultimate effect of TSP1 on capillary morphogenesis. In fact, the region of TSP1 responsible for the activation of TGF-ß1 appears to be distinct from the antiangiogenic domain (15). Whether the binding of TGF-ß provides an additive effect to the angiogenic properties attributed to TSP1 remains to be determined.

An important question is whether endogenous TSP1 blocks blood vessel formation during normal physiological and pathological responses in which angiogenesis is a predominant feature. Since recurrent stimulation and inhibition of capillaries are important characteristics of the cycling human endometrium, we studied the expression and distribution of TSP1 in this tissue. Our findings indicate that TSP1 is expressed in the human endometrium during the secretory phase, coincidentally with the suppression of angiogenesis, and that TSP1 is regulated by progesterone in human endometrial stromal cells, but not in human dermal fibroblasts. Moreover, the TSP1 produced by progesterone-stimulated endometrial stromal cells specifically inhibited the directed

migration of endothelial cells. These results are consistent with the recognized function of TSP1 as an inhibitor of angiogenesis that might be regulated by progesterone specifically in the secretory phase of the human endometrial cycle.

Methods

Materials. Paraformaldehyde, Ficoll-400, polyvinylpyrrolidone, salmon testicular DNA, dextran sulfate, RNAse A, progesterone, and 17-ß-estradiol were purchased from Sigma Chemical Co. (St. Louis, MO). RU-486 ((11ß,17ß)-11-[4-(dimethylamino)phenyl]-17-hydroxy-17-(1 -propynyl estra-4,9-diene-3-one) was the generous gift of Dr. Bruce Kessel (Division of Obstetrics and Gynecology, Beth Israel Hospital, Boston, MA); growth factor-free BSA was a gift from Elaine Raines (Dept. of Pathology, Univ. of Washington, Seattle, WA). Anti-TSP monoclonal antibodies were obtained from Boehringer Mannheim (Indianapolis, IN). Polyclonal anti-human platelet TSP1 has been described (9). Anti-proliferating cell nuclear antigen (PCNA) antibodies were purchased from Becton Dickinson (San Jose, CA), and anti-progesterone receptor and anti-estrogen receptor antibodies, from Affinity Bioreagents, Inc. (Neshanic Station, NJ). Trypsin-EDTA, collagenase, phenol-red free medium, and antibiotics were from Gibco Laboratories (Grand Island, NY). Charcoal-filtered fetal calf serum was supplied from Cocalico Biologicals (Reamstown, PA). The random primed DNA-labeling kit and the [^{32}P]-dCTP (3,000Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). L-[2,3,4,5-^{3}H]-proline and [^{125}I]-protein A were purchased from NEN (Boston, MA). Nylon membranes (Nytran) were purchased from Schleicher and Schuell (Keene, NH). All other reagents used in this study were of the highest purity available from Fisher Biochemicals (Pittsburgh, PA).

Immunocytochemistry and in situ hybridization. Endometrial samples were obtained from hysterectomy specimens from women who underwent surgery for benign indications or conditions that did not compromise the endometrial layer. The age of the patients varied from 29-74 years. Twenty-two specimens of endometrium were obtained in the proliferative phase, sixteen from the secretory phase, and eight from postmenopausal patients. The phase of the menstrual cycle was determined by histological examination of the tissue. The specimens were obtained from the Pathology Departments of the University of Washington, School of Medicine, Seattle WA and from Beth Israel Hospital, Boston, MA. The use of the tissue was approved by the Institutional Human Studies Experimentation Committees at both Institutions.

For immunocytochemistry, the tissues were immersed in methyl-Carnoy's fixative (60% methanol, 30% chloroform, 10% acetic acid). For in situ hybridization, fixation was done in 4% paraformaldehyde. Most of the specimens were dehydrated and embedded in paraffin; a few samples were also analyzed as frozen sections. Sections were rehydrated and processed by an avidin-biotin-immunoperoxidase method (Vectastain ABC; Vector Labs, Burlingame, CA), as

previously described (19). The primary antibodies included: (a) affinity-purified rabbit anti-TSP-1 (10μg/ml) (9); (b) mouse anti-human TSP1 antibody, and (c) mouse anti-PCNA.

In situ hybridization was performed as previously described (20). The cDNA fragment used as a template for in situ hybridization was a 1.3kb *Eco*RI/*Eco*RI fragment of human TSP1 cloned into the vector pGEM-2 in an anti-sense orientation relative to SP6. Digestion with *Ear*I provided an antisense riboprobe of 395 bp, when the SP6 polymerase was used, and digestion of the same template with *Nar*I provided a 315 bp sense riboprobe, when T7 RNAse polymerase was used. The riboprobes comprised 132 amino acids from the amino terminus of the TSP1 protein. This region is only 33% identical to TSP2 at the nucleotide level, and the resulting riboprobes have been shown not to cross-hybridize with other members of the TSP family (20). In addition, when hybridization with a TSP2-specific riboprobe was performed on the same samples, patterns of expression between TSP1 and TSP2 did not overlap (data not shown). The sections were hybridized overnight at 53°C. Slides were coated with Kodak NTB2 emulsion (Eastman Kodak, Rochester, NY) and were exposed in the dark for 2 weeks at 4°C. Hybridization with sense probes served as negative controls.

Both immunocytochemistry and in situ hybridization procedures for experimental and control specimens were done under identical conditions. Two different investigators independently evaluated the degree and pattern of immunostaining.

Isolation of cells and tissue culture. Endometrial samples were minced into 1-2 mm^3 pieces under sterile conditions in a laminar flow hood. Fragments were placed into 48 mm^2 COSTAR™ plates. Stromal cells migrated out of the explants after 2-3 days of plating, and explants were removed after 7 days. Cells were grown to confluency in phenol-red free DMEM containing 10% charcoal-filtered fetal calf serum, supplemented with 10nM 17-ß-estradiol, 10μM progesterone, and 100μg/ml penicillin G/streptomycin sulfate. At confluence, cultures were incubated in serum-free medium for 2 days and were treated with steroids, growth factors, or serum. Human dermal fibroblasts were purchased from American Type Culture Collection (Rockville, MD). Dermal fibroblasts, isolated from a 33 year-old woman (strain DF-33a), were grown under the same conditions used for stromal cells and used in parallel experiments with stromal cells for purpose of comparison. To confirm the results obtained with the dermal strain DF-33a, six additional dermal primary cultures isolated from different patients (ages 35-56) were also tested and identical results were obtained.

BAE cells were isolated as previously described (20).

Identification of progesterone receptors in stromal cells. The presence of progesterone receptors in cultured stromal cells was verified by immunocytochemistry and by reverse transcriptase followed by polymerase chain reaction. For immunocytochemistry, stromal cells at passage 3 were plated on glass coverslips, grown until 80% confluent, and fixed in methyl-Carnoy's solution for 10 min. Cultures were then washed in PBS, blocked in 1% goat serum, and incubated with 30 µg/ml of anti-progesterone receptor antibody. Detection of immunocomplexes was accomplished with an FITC-conjugated anti-mouse IgG. Negative controls included (a) incubation of stromal cells with secondary antibody alone, and (b) immunocytochemistry on human dermal fibroblasts.

To confirm the expression of progesterone receptors by stromal cells, we subjected RNA to reverse transcriptase followed by PCR analysis. The primers used for the analysis included: 5' CAAAACTTCTTGATAACTTGCA 3' (forward primer) and 5'CACTTTTTATGAAAGAGAAGGG 3' (reverse primer); oligonucleotides were made by Integrated DNA Technologies (Coralville, IA). We conducted a computer-assisted search (GenBank) of the above progesterone receptor oligo-DNA sequence and found that only progesterone receptor mRNAs of human, mouse, and rat, but not other known sequences, would be expected to hybridize with the above antisense probe. Total RNA from: (a) human cycling endometrium, (b) cultured stromal cells isolated from a 35 year-old human endometrium, (c) cultured stromal cells isolated from a 37 year-old human endometrium, and (d) cultured dermal fibroblasts, were subjected to reverse transcriptase for the generation of first-strand cDNAs. These cDNAs were subsequently amplified by PCR. The reaction resulted in the predicted 179bp band. The products were analysed on a 1.5% agarose gel.

Metabolic labeling. Cultures were incubated with 50 µCi/ml of L-[2,3,4,5-^{3}H]-proline for 16h. The conditioned medium was centrifuged to remove cell debris, and proteinase inhibitors were added to produce the following final concentrations: 6.3 mM *N*-ethylmaleimide; 2 mM phenylmethylsulfonyl fluoride; 25 mM EDTA, and 5 µg/ml pepstatin A. Medium proteins were precipitated in 10% trichloroacetic acid and were resolved by SDS-PAGE. The cell layers were scraped into PBS containing the same proteinase inhibitors and were sonicated prior to analysis. In independent experiments, the amounts of samples for SDS-PAGE were normalized according to total protein levels, cell number, or total counts. Since similar results were obtained despite these different criteria, we have presented data from experiments in which the amount of each sample was normalized to cell number (medium from 500,000 cells/lane). Western blots of the same samples were performed as previously described (9); immunocomplexes were detected with [^{125}I]-protein A and autoradiography.

Northern analysis. Total RNA was purified from cells by guanidinium-isothiocyanate extraction (22). Samples were subjected to electrophoresis on a denaturing 1.2% agarose gel. Northern blots were hybridized with [^{32}P]-labeled cDNA probes . The TSP1 probe corresponded to a 1.3 kb *Eco*RI-*Eco*RI fragment from human TSP1 cDNA (9); the 28S rRNA probe was a *Eco*RI-*Eco*RI 280 bp fragment from bovine cDNA (23). Membranes were exposed to Kodak XAR-5 film, and densitometric analysis of autoradiograms exposed within the linear response range of the film was performed with a scanning densitometer (Personal Densitometer, Molecular Dynamics). Alternatively, we used phosphoImager screens to quantify some of the Northern blots (Molecular Dynamics). The 28S signal was utilized to correct for loading and transfer inequalities.

The densitometric values were expressed as a percentage of paired samples of non-treated controls (always tested on the same Northern blot), after correction for loading differences with the 28S signal, and are shown as mean ± SEM. Statistical studies were carried out by analysis of variance followed by the Newman-Keuls test for multiple comparisons (24). Differences were considered statistically significant at $P<0.01$.

Migration assays

BAE cells at passages 4-8 were maintained in 10% FCS until confluence. Cultures were incubated in serum-free DMEM containing 0.1% growth-factor-free BSA for 48h, removed from the dishes with trypsin, and replated at a concentration of 10^4 cells / well on the surface of a 8µm filter (Nucleopore Corporation, Pleasanton, CA) placed in a modified Boyden chamber. The opposite side of the filter was coated with 50µg/ml of vitrogen (Celtrix) and blocked with 1% BSA. Cells were allowed to attach and spread. After 2h at 37°C, the following solutions, all containing 10ng/ml bFGF, were added to the lower chamber: (1) stromal cell-conditioned media; (2) stromal cell- conditioned media containing 20µM progesterone; (3) stromal cell-conditioned media after exposure to progesterone for 12h; (4) DMEM containing 10µg TSP1; (5) stromal cell-conditioned media after exposure to progesterone for 12h and subsequent incubation with anti-TSP1 antibodies (20µg/ml) for 6h; (6) DMEM containing anti-TSP1 antibodies (20µg/ml). The conditioned media were obtained from stromal cultures incubated for a total of 24h in the absence of FCS. Treatment with progesterone was performed during the second 12h of incubation. Addition of bFGF was necessary to stimulate migration; in the absence of bFGF, no significant migration was detected in any of the samples. Results were expressed as percent of migration after subtraction of background (migration in BSA) and are reported as percent of control ±SE .

Migration was allowed to proceed for 4h; filters were subsequently fixed for 30 min in 4% paraformaldehyde and were stained with 1% toluidine blue. To obtain statistically significant values, we counted 5 random fields under the 10X objective. Experiments were performed in

triplicate and were repeated four times. The blocking experiment with anti-TSP antibodies was performed twice. Experiments conducted in the presence of TSP1 were done twice, in triplicate. Two different preparations of TSP1 were evaluated: one was prepared in our laboratory, and the other was provided by Dr. Joanne Murphy-Ullrich (Dept. of Pathology, University of Alabama, Birmingham). The latter TSP1 had been stripped of contaminating traces of TGF-ß1.

Results

Endometrial specimens were obtained from 46 women, 38 of fertile age and 8 post-menopausal. From the cycling specimens, twenty-two biopsies were classified as proliferative and sixteen as secretory, based on histological criteria. Serial sections from each sample were examined by immunocytochemistry for expression of PCNA and TSP1. In most cases there was an inverse correlation between the presence of TSP1 protein and the proliferation of endometrial cells (Fig. 1A-D). Furthermore, within the histological classification of the specimens (proliferative, secretory, or inactive), the expression of TSP1 was restricted to the secretory phase. Tissues from the early proliferative phase showed low, or no, immunoreactivity. Table I shows results from all the specimens examined.

We observed TSP1 protein predominantly in the basement membranes of glands, and in small blood vessels and capillaries (Fig. 1D-H, arrows). Identification of vessels with an anti-CD34 antibody in serial sections demonstrated that only a subset of capillaries was reactive with anti-TSP-1 antibodies (data not shown). Moreover, immunostaining with PCNA IgG indicated that the presence of TSP1 protein did not, in most instances, correlate with proliferating endothelial cells. TSP1 expression was predominant in the functionalis, which corresponds to the upper 2/3 of the endometrium, and the number of positive microvessels was higher in the subepithelial stroma (Fig. 1E). Diffuse staining for extracellular TSP1 was also detected in the stroma, particularly in the subepithelial layer (Fig. 1E). Staining of the stroma was seen only in frozen sections (Fig. 1E-F). No staining was detected with preimmune serum in either proliferative or secretory endometrium (Fig. 1I).

We also performed in situ hybridization on similar sections to localize TSP1 transcripts. Results were consistent with the immunocytochemical data. Low levels were detected in proliferative endometrium (Fig. 2A), in contrast to the abundant expression of TSP1 mRNA in secretory endometrium (Fig. 2 B). TSP1 mRNA was also observed in endothelial cells, but appeared more abundant in stromal cells of the human endometrium. Whereas high levels of transcript were observed in the functional layer, only background levels, equivalent to the intensity observed during the proliferative phase, were seen in the basal layer (Fig. 2 C-F). Transcripts were present chiefly in stromal cells from the upper 1/3 of the endometrium (Figure 2 F), a result consistent with the location of the protein by immunocytochemistry (Fig. 1E).

Temporal restriction of TSP1 expression was further confirmed by Northern blot analysis of total RNA samples isolated from proliferative, secretory, and post-menopausal endometrium. TSP1 mRNA was detected in all samples; however, a 3.5-4.2 fold increase in signal was observed in samples from the secretory phase in comparison to samples from the proliferative phase (Fig.

3). These findings, together with our immunohistochemical and *in situ* hybridization data, indicated that the synthesis of TSP1 might be regulated by steroids.

To study hormonal regulation of TSP1, we isolated endometrial stromal cells and developed conditions for their maintenance in culture. Stromal endometrial fibroblasts exhibited morphological features similar to those of dermal fibroblasts (Fig. 4A). Stromal cells showed high levels of TSP1 by immunocytochemistry (Fig. 4B) and Western blot analysis (not shown). We examined initially the expression of progesterone and estrogen receptors in cultured stromal cells by immunocytochemisty and RT-PCR. Both receptors were present in early-passage cells; however, we observed a gradual decrease in the percentage of positive cells with time in culture that was independent of the presence of estradiol and progesterone in the media. This decrease was not dependent on the endometrial phase (proliferative or secretory) at the time of cell isolation. We examined systematically the levels of progesterone receptor from passages 2-9 and found that, in most primary cultures, steroid receptors were not detected after passage 7. Therefore, experiments that examined the regulation of TSP1 by steroids were performed on stromal cells of passages 2-4. Expression of the progesterone receptor is shown by immunocytochemistry in Figure 4C. The presence of the progesterone receptor was also confirmed by RT-PCR (Figure 4D). The results indicate that cultured stromal fibroblasts (Figure 4D, lanes 3 and 4), but not dermal fibroblasts (Figure 4D, lane 5), transcribe mRNA for the progesterone receptor.

In fibroblasts and smooth muscle cells, TSP1 mRNA is increased by serum and PDGF (25, 26). TSP1 mRNA was likewise induced in endometrial-derived stromal cells by serum and PDGF (Fig. 5A); however, in these cells, TSP1 mRNA was also elevated by progesterone (Fig. 5A, B). Stimulation by progesterone was similar to the effect mediated by PDGF, with increases of 3.1- and 3.6-fold, respectively. The regulation of TSP1 by progesterone was cell-specific, since dermal fibroblasts cultured under identical conditions did not show an increase in steady-state levels of TSP1 mRNA (Fig. 5B). These experiments were performed on a total of seven independent isolates of human dermal fibroblasts, with identical results. In contrast to the effect mediated by progesterone, TSP1 mRNA levels were not increased by treatment with 17-ß-estradiol (Fig. 5C). Steady-state levels of TSP1 mRNA in human smooth muscle cells were also not altered during culture in the presence of progesterone and 17-ß-estradiol (Fig. 5C).

The specificity of the response of TSP1 to progesterone was tested with the progesterone analog RU-486, which blocks the function of the progesterone receptor (27,28). After confluent cultures of stromal cells were incubated with RU-486 at concentrations of 1 and 50 µM, we saw no change in steady-state levels of TSP1 mRNA (Fig. 5D). Preincubation of cultures with 50 µM RU-486, followed by progesterone, suppressed the stimulatory effect mediated by progesterone

alone (Fig. 5D). This experiment was performed 3 times with 2 independent isolates of stromal cells; in each case, identical results were obtained.

The regulation of TSP1 mRNA by progesterone was concentration-dependent. Stromal cells were grown in steroid-depleted media (Methods), cultured until confluence, and incubated under serum-free conditions for 48h. This protocol reduces the levels of TSP1 mRNA to what we have termed "baseline". Maximum induction of TSP1 mRNA was seen with 10-20 μM progesterone, although induction of mRNA was detected after incubation with as little as 0.5 μM progesterone (Fig. 6). The concentrations of progesterone used in these experiments corresponded to normal physiological levels, which are known to be between 5 and 30 μM (29). Further increases in TSP1 were not apparent at concentrations of progesterone above 20 μM (Fig. 6). The maximum increase in steady-state levels of TSP1 mRNA was 4.2-fold in human stromal endometrial cells after 6h of exposure to progesterone (Fig. 6). These experiments were performed a total of 7 times with 4 strains of stromal cells isolated from both proliferative and secretory endometrium. Northern blots were quantified by densitometry. Signals from various samples were normalized to those corresponding to the 28S rRNA and were expressed as percent of control (fold increase; a concentration of 0 was designated baseline). Figure 6B illustrates the quantification of 5 independent experiments with 3 different cell strains.

We also performed time-course experiments with a constant concentration of progesterone. Confluent cultures of stromal cells, subjected to 48h of serum-deprivation, were incubated with 10 μM progesterone and were harvested at 2, 4, 6, 8, and 24h. Increased levels of TSP1 mRNA were detected after 4h; however, maximal induction of 3.8-fold over control occurred after 8h (Fig. 7). After 24h of incubation with progesterone, levels of TSP1 mRNA were below control values (Fig. 7). This result was reproduced in 4 independent experiments and was also seen at 36h and 48h. The data indicate that the regulation of TSP1 by progesterone might be biphasic, with an initial stimulation followed by an inhibition. An alternative explanation is that extended exposure to this hormone decreases the number of progesterone receptors (29) and results in suppression of the stimulatory effect.

Treatment with progesterone resulted in increased levels of secreted TSP1 protein. Cultures of stromal cells were incubated with 10 μM progesterone and L-[2,3,4,5-^{3}H]-proline for 12h. Some cultures were also treated with RU-486 for 4h, prior to the addition of progesterone and L-[2,3,4,5-^{3}H]-proline. Proteins from conditioned media were subjected to electrophoresis, as shown in Fig. 8A. Although changes in TSP1 were not evident by SDS-PAGE, quantification of Western blots revealed a 2.5-3.5 increase in the levels of TSP1 after exposure of cells to progesterone (Fig. 8B). RU-486 blocked the effect mediated by progesterone (Fig. 8B).

To determine whether the increase in TSP1 levels induced by progesterone were sufficient to affect endothelial cell function, we tested the effect of media from stromal cultures on the migration of BAE cells. The experiments were performed in the presence of bFGF, a known stimulator of endothelial cell migration. Conditioned media from stromal cells previously incubated with progesterone for 12h inhibited bFGF-mediated migration of BAE cells by 32% (Fig. 9, column 3). Conditioned media from untreated cultures did not show a statistically significant effect on the migration of BAE cells. The inhibition of migration was blocked after neutralizing antibodies against TSP were preincubated with conditioned media from the treated cultures (Fig. 9, column 5). At the concentration used in these experiments, anti-TSP antibodies alone did not alter the migration of endothelial cells. DMEM containing TSP1 at a concentration comparable to that induced by progesterone produced a similar effect on the inhibition of migration (Fig. 9 column 4). These results indicate that the inhibition of migration of endothelial cells by cultured medium from stromal cells exposed to progesterone could be attributed to TSP1.

Discussion

The effect of TSP1 on the inhibition of blood vessel formation has been demonstrated both in vivo and in vitro (7-12). Experiments in vivo have focused on the ability of TSP1 to suppress the angiogenic response mediated by bFGF in the corneal pocket assay (8, 11); nevertheless, the role of TSP1 as an intrinsic regulator of blood vessel formation at sites of angiogenesis, such as wound repair, tumors, and/or endometrial/ovarian cycling, remains unclear. In this study, we utilized the human endometrium, a site of recurrent angiogenesis, to ask (a) whether TSP1 synthesis occurred in the endometrium, (b) if TSP1 expression was associated with the end of the proliferative phase/begining of the secretory phase in normal endometrium, a time when capillary formation is inhibited, and (c) whether TSP1 expression was affected by steroid hormones. We found that TSP1 was expressed at high levels in the functional layer of the endometrium. The peak of expression occurred during the secretory phase and was coincident with the cessation of capillary growth. Finally, we found that TSP1 mRNA and protein were regulated by progesterone and that the increased levels were sufficient to inhibit the migration of endothelial cells.

The human endometrium undergoes cyclic, hormonally-dependent changes in proliferation, differentiation, sloughing, and repair. During this process, the need for additional vasculature is constantly imposed by the cyclic differentiation of transient structures and by the recurrent repair of damaged tissues. It has been suggested, but not proven, that the initial wave of capillary proliferation is under the control of steroid hormones (29-31). It is assumed, therefore, that the repair of the endometrial vasculature is mediated by angiogenic and anti-angiogenic factors that are either directly or indirectly inducible by hormones.

An association between angiogenic activity and steroid hormones was initially suggested by experiments in which endometrial tissue was transplanted to the anterior chamber of the monkey eye (32) and to the hamster cheek pouch (33). In both models, the extent of vascularization and incidence of bleeding were influenced by ovarian steroids. These experiments support the concept that the angiogenic response in the primate endometrium is, at some level, dependent on the presence of estrogen and progesterone. Similarly, the regulation of angiogenic inhibitors is likely to be controlled by steroids. Since TSP1 was detected in the human endometrium in a specific phase of the endometrial cycle, we examined the regulation of TSP1 by both estrogen and progesterone. Treatment of stromal cells with physiological levels of 17-ß-estradiol did not increase TSP1 expression; however, incubation with progesterone resulted in an approximately 4-fold increase in steady-state levels of TSP1 mRNA. This response was dependent on the presence of progesterone receptor, since later-passage cells lacking the receptor did not show an increase in TSP1 mRNA levels. The effects mediated by progesterone occurred at concentrations considered

to be available to cells in vivo. Moreover, the increase in TSP1 mRNA by progesterone appeared to be cell-specific.

Steroid hormones trigger the binding of specific receptor proteins to promoter/enhancer elements of target genes, an interaction resulting in the activation of transcription (34-35). Analysis of the mouse (36) and human (this report) TSP1 genes revealed the presence of a palindromic consensus sequence that has been reported to interact with the progesterone receptor (35). In the human TSP1 gene we found two progesterone-responsive elements in the promoter. It remains to be seen whether these sites are functional and responsible for the progesterone-inducible response.

Although the possibility that the TSP1 gene might be responsive to steroids had not been explored, previous studies were suggestive of such regulation (37-39). High levels of TSP1 have been reported in other organs sensitive to steroid hormones, such as the mammary gland under both normal and pathological conditions (37). TSP1 protein has also been detected in milk, other breast secretions, and in some types of mammary cystic fluids (40). Interestingly, a study on the kinetics of TSP accumulation in human milk has shown temporal variations which could be due to hormonal regulation. TSP was detected in the initial "aqueous phase" of milk secretion and its levels subsequently fell during the transition to mature milk (38). More recently, changes in plasma TSP1 have also been identified in patients with gynecological malignancies (41).

A number of laboratories have reported inhibition of angiogenesis by steroids (42,43). For example, glucocorticoids cause regression of hemangiomas if injected directly into the lesion or if given at high doses systemically (44). Since the glucocorticoid and the progesterone receptors share the same nucleotide binding sequence (45), it would be interesting to determine whether the inhibition of blood vessel formation mediated by steroids in these tumors has any correlation with the secretion of TSP1.

The present study did not address the possible role of TSP2 in the inhibition of endometrial angiogenesis. TSP2, a second member of the TSP family, shows a high sequence similarity to TSP1 (3). Both proteins contain the procollagen homology region and type I repeats and have been considered to share anti-angiogenic properties (11). We were unable to detect TSP2 mRNA in the human endometrium (data not shown) or in stromal endometrial cells in vitro. Although the proteins have common structural features and remarkable amino acid similarity, diverse sites of expression (20, this report) and differences in gene regulation (46,47) are indicative of independent functions. Furthermore, the lack of TSP2 in the human endometrium provides compelling evidence that, at least in this organ, the inhibition of capillary formation is dependent on TSP1.

What are the functional consequences of an increase in the levels of TSP1 during the secretory phase of the endometrial cycle? Our data show that the induction of TSP1 by progesterone is sufficient to inhibit the migration of endothelial cells in vitro. We therefore propose that, in the human endometrium, TSP1 antagonizes the effect of angiogenic stimulators that induce endothelial cell migration during angiogenesis.

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Table I. Correlation between endometrial phase and presence of TSP1 in human endometrium.

Patient	Age	Endometrial phase	Diagnosis	TSP1	PCNA
1.	34	proliferative	n.d.	-	+++
2.	36	proliferative	n.d.	-	+
3.	36	proliferative	cervical cancer	-	++
4.	37	proliferative	ovarian cancer	+	+++
5.	39	proliferative	cervical cancer	+	+++
6.	41	proliferative	leiomyoma	-	+++
7.	41	proliferative	leiomyoma	-	++
8.	42	proliferative	leiomyoma	+	+++
9.	42	proliferative	n.d.	+	++
10.	42	proliferative	leiomyoma	-	+++
11.	43	proliferative	leiomyoma	+	+++
12.	43	proliferative	leiomyoma	+	+++
13.	44	proliferative	leiomyoma	-	+++
14.	44	proliferative	cervical cancer	-	+++
15.	45	proliferative	cervical cancer	+	+++
16.	45	proliferative	leiomyoma	-	++
17.	46	proliferative	ovarian cancer	-	+++
18.	47	proliferative	cervical cancer	-	+++
19.	49	proliferative	n.d.	+	+++
20.	48	weakly proliferative	endometrial cancer	-	+
21.	37	mid-secretory	n.d.	++	-
22.	29	secretory	leiomyoma	+	+++
23.	30	secretory	leiomyoma	++	+
24.	31	secretory	cervical cancer	+++	-
25.	33	secretory	cervical cancer	+	-
26.	32	secretory	leiomyoma	+	-
27.	37	secretory	ovarian cancer	+++	-
28.	37	secretory	leiomyoma	++	-
29.	39	secretory	ovarian cancer	++	-
30.	40	secretory	leiomyoma	+++	++
31.	42	secretory	cervical cancer	+++	-
32.	42	secretory	leiomyoma	+++	+
33.	42	secretory	leiomyoma	+	-
34.	44	secretory	n.d.	-	-
35.	47	secretory	leiomyoma	+++	-
36.	47	secretory	n.d.	+++	+
37.	48	secretory	leiomyoma	++	+
38.	49	secretory	ovarian cancer	++	+
39.	44	inactive	leiomyoma	-	-
40.	51	inactive	leiomyoma	-	+
41.	53*	inactive	leiomyoma	+	+
42.	55	inactive	cervical cancer	-	-
43.	57	inactive	cervical cancer	-	+
44.	62	inactive	leiomyoma	-	-
45.	74	inactive	n.d.	-	
46.	54	non-phasic	endometritis	-	-

Identification of proliferating cells was performed with a PCNA (proliferating cell nuclear antigen) antibody followed by biotinylated secondary IgG and avidin-biotin-peroxidase. The number of proliferating cells and histological characteristics were used to classify the phase of the endometrial cycle.

*estrogen treatment; n.d., not determined; -, none; +, low; ++, moderate; +++, high

FIGURE LEGENDS

Figure 1. Expression of TSP1 protein in the proliferative and secretory human endometrium.

Sections were incubated for 1h with anti-PCNA IgG (**A** and **B**) or with anti-TSP1 antibody (**C - H**). Immunocomplexes were identified by an avidin-biotin-peroxidase procedure. From the histological profile and relative number of proliferating cells, the proliferative phase (**A** and **C**) and secretory phase (**B, D,** and **E-I**) of the endometrial cycle can be identified. Arrows in **A** and **B** indicate nuclei of proliferating cells. No immunoreactivity for TSP1 was detected in proliferative endometrium (**C**), in contrast to the secretory endometrium (**D**), in which vessels reactive to anti-TSP1 IgG were identified (arrows). In the secretory endometrium, TSP1 was also detected in the subepithelial stroma (**E**). Higher magnification showed that TSP1 was located extracellularly in the stroma and in the basement membranes of vessels and glands (**F,** arrows). In the basal endometrium, TSP1 was predominantly in the basement membrane of some blood vessels (**G** and **H**). Two vessels are shown in **H**, one containing TSP1 (closed arrow) and one in which TSP1 was not detected (open arrow). A negative control with preimmune serum is shown in **I.** Asterisks identify glands; **e,** superficial epithelium.

Bars: **A-D,** 100μm; **E**, 75μm; **F** and **H**, 25μm; **G** and **I**, 125μm

Figure 2. Localization of TSP1 transcripts in the human endometrium by in situ hybridization.

All sections were hybridized with anti-sense riboprobes for human TSP1. (**A**) Proliferative phase; (**B-F**) secretory phase. Note the gradual decrease of TSP1 signal from the stratum functionalis (*sf* in **C** and brackets in **D** and **E**) to the stratum basalis (*sb*) and the myometrium (*m*). TSP1 transcripts were predominantly in stromal cells (**F,** arrows). Asterisks in **A-C** identify glands, arrows in **C** indicate the transition of sf to sb and sb to m; **e**, epithelium. Bar= 100μm

Figure 3. Levels of TSP1 mRNA in human endometrial samples.

Hybridization of Northern blots was performed with a human TSP1 cDNA probe. After scanning densitometry, the blot was stripped and hybridized with a 28S rRNA probe. Probes are indicated on the left. Lanes: (**1**) post-menopausal endometrium (65 years old); (**2**) inactive endometrium (40 years old); (**3**) endometrium from proliferative phase (25 years old); (**4**) endometrium from secretory phase (37 years old); (**5**) endometrium from late secretory phase (41 years old); (**6**) human smooth muscle cells.

Figure 4. Human endometrial stromal cells in vitro.

Isolation and culture of human endometrial stromal cells are described in Materials and Methods. (**A**) Phase-contrast micrograph of stromal cells; (**B**) immunocytochemical detection of TSP1 (arrow indicates a reactive cell); (**C**) immunodetection of progesterone receptors in cultured endometrial stromal cells (arrows); (**D**) identification of 179 bp progesterone receptor fragment produced by RT-PCR. Lanes: (1) 123 bp DNA ladder; (2) RT-PCR of human endometrium RNA; (3) RT-PCR of cultured stromal cells isolated from human endometrium (35 year-old patient); (4) RT-PCR of cultured stromal cells isolated from human endometrium (37 year-old patient); (5) RT-PCR of cultured dermal fibroblasts. Arrow indicates the migration of the predicted 179 bp fragment.

Figure 5. Stimulation of TSP1 mRNA by serum, PDGF-AB, progesterone, and estradiol.

A. Confluent cultures of stromal cells were maintained without serum for 48h. Cells were subsequently incubated with (1) no serum; (2) 5% charcoal-filtered FCS; (3) buffer for PDGF-AB; (4) 10 ng/ml PDGF-AB; (5) buffer for progesterone; (6) 10 μM progesterone for 8h. Total RNA was extracted, subjected to electrophoresis, transferred, and hybridized with probes for TSP1 and 28S rRNA.

B. Confluent cultures of dermal fibroblasts and endometrial stromal cells from women of the same age were treated with 10 μM progesterone for 8h. Northern blots were hybridized with probes for TSP1 and 28S rRNA. Lanes: (1) dermal fibroblasts and buffer; (2) dermal fibroblasts incubated with 10μM progesterone; (3) stromal cells and buffer; (4) stromal cells incubated with 10μM progesterone.

C. Confluent cultures of stromal cells (1, 2) and smooth muscle cells (3-5) were treated with 10 nM 17-ß-estradiol (2 and 4), 10 μM progesterone for 8h (5), or DMEM alone (1 and 3). Northern blots were hybridized with probes for TSP1 and 28S rRNA.

D. Cultures of endometrial stromal cells previously incubated in serum- and steroid-free DMEM for 48h were treated with (1) DMEM alone; (2) 50 μM RU-486; (3) 20μM of progesterone for 4h, or (4) 50 μM RU-486 for 4h, followed by 20μM of progesterone for 4h. Resulting RNA blots were hybridized with probes for TSP1 and 28S rRNA.

Figure 6. Concentration-dependent response of TSP1 mRNA to progesterone

A. Cultures of human stromal cells were incubated for 8h with increasing concentrations of progesterone. Total RNA was purified and resolved on an agarose gel. Hybridization was

performed with a human cDNA TSP1 probe and with a 28S rRNA probe for normalization of loading and transfer efficiency.

B. Five independent experiments were scanned by densitometry, normalized to the rRNA signal for 28S rRNA, and expressed as fold increase. A concentration of 0 was defined as the baseline (mean ± SE).

Figure 7. Temporal induction of TSP1 mRNA by progesterone

A. Human stromal cells were exposed to 10μM progesterone for the times indicated (0 to 24h). 7 μg of total RNA was resolved on a 1.2% agarose gel, transferred to Nytran membranes, and hybridized with a TSP1 cDNA probe and a 28S rRNA cDNA probe.

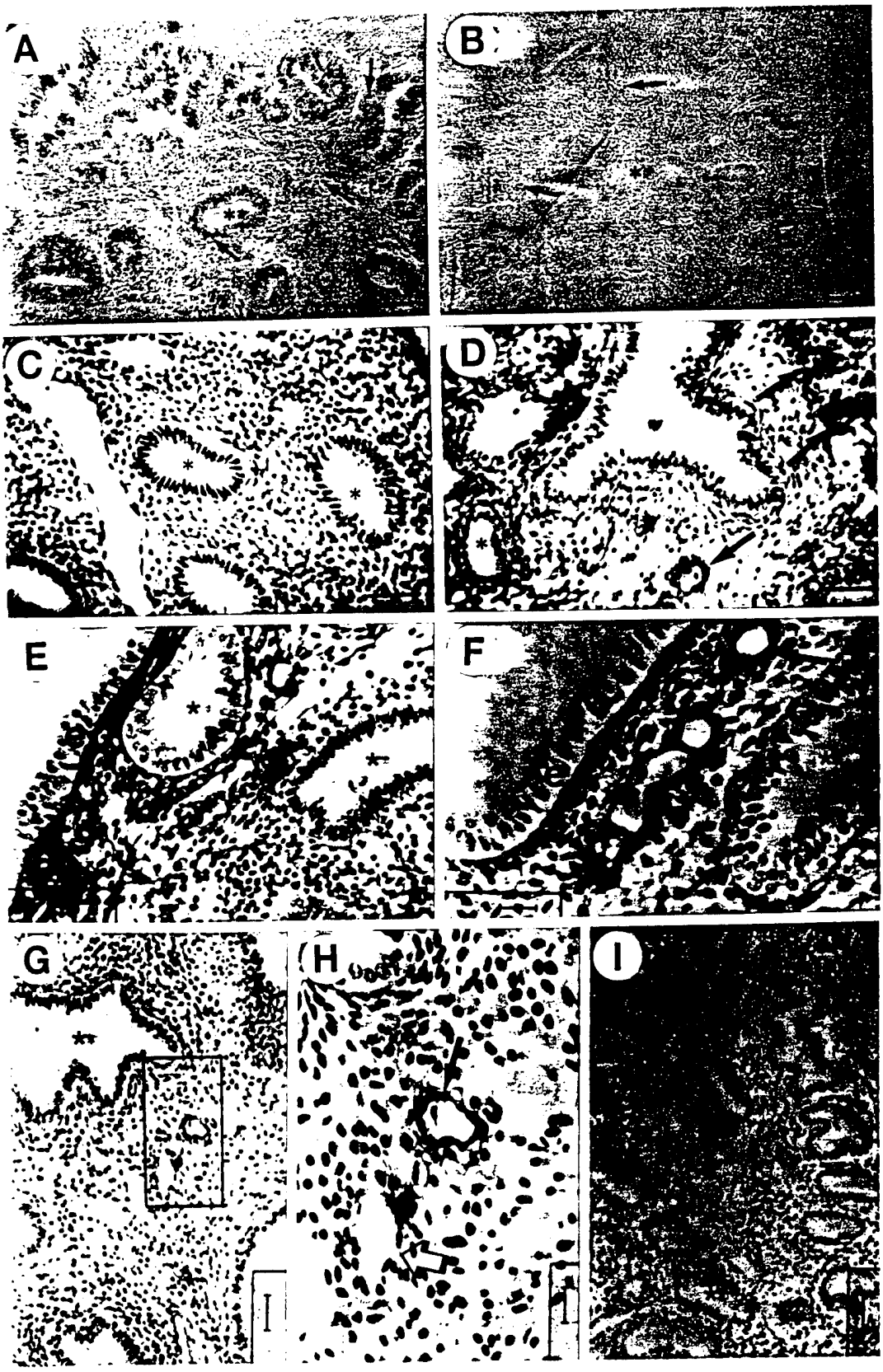
B. Blots from four independent experiments were scanned by densitometry, normalized to the corresponding signal for 28S rRNA, and expressed as fold increase in the histogram. The absorbance at time 0 was defined as 1.

Figure 8. Exposure of human stromal fibroblasts to progesterone increases the level of secreted TSP1 protein.

Cultures of stromal fibroblasts were incubated for 4h with: (1) DMEM alone; (2) 10 μM progesterone; (3) 50 μM RU-486 for 4h followed by 10 μM progesterone; (4) 20 μM progesterone. After the incubation period, 50 μCi/ml [2, 3, 4, 5, ^{3}H]-proline was added to each culture. **(A)** Radiolabeled proteins, secreted by 500,000 cells/sample, were resolved on a 4%/8% SDS-PAGE gel under reducing conditions and were visualized by autoradiography. **(B)** Western blot of the same samples shown in (A), after incubation with anti-TSP antibodies followed by [^{125}I]-protein A. **MwS**, Molecular weight standards.

Figure 9. Effect of conditioned media on the migration of endothelial cells.

Endothelial cells were plated on a gelatinized filter (pore=8μm). Conditioned media were collected from stromal cultures incubated for 24h in the absence of FCS. Migration of endothelial cells was evaluated 4h after exposure to the following solutions, all of which contained 10ng/ml bFGF: (1) stromal cell-conditioned media (SCM); (2) DMEM containing 20 μM progesterone; (3) stromal cell- conditioned media from cultures exposed to 20 μM progesterone for 12h; (4) DMEM containing 10μg TSP1; (5) media from (2) incubated with anti-TSP1 antibodies (20 μg/ml); (6) DMEM containing anti-TSP1 (20 μg/ml) alone. Results are expressed as % of control ± SE. 0 (or background migration) was equivalent to 5 cells/field; 100% migration was equivalent to 65 cells/field


A
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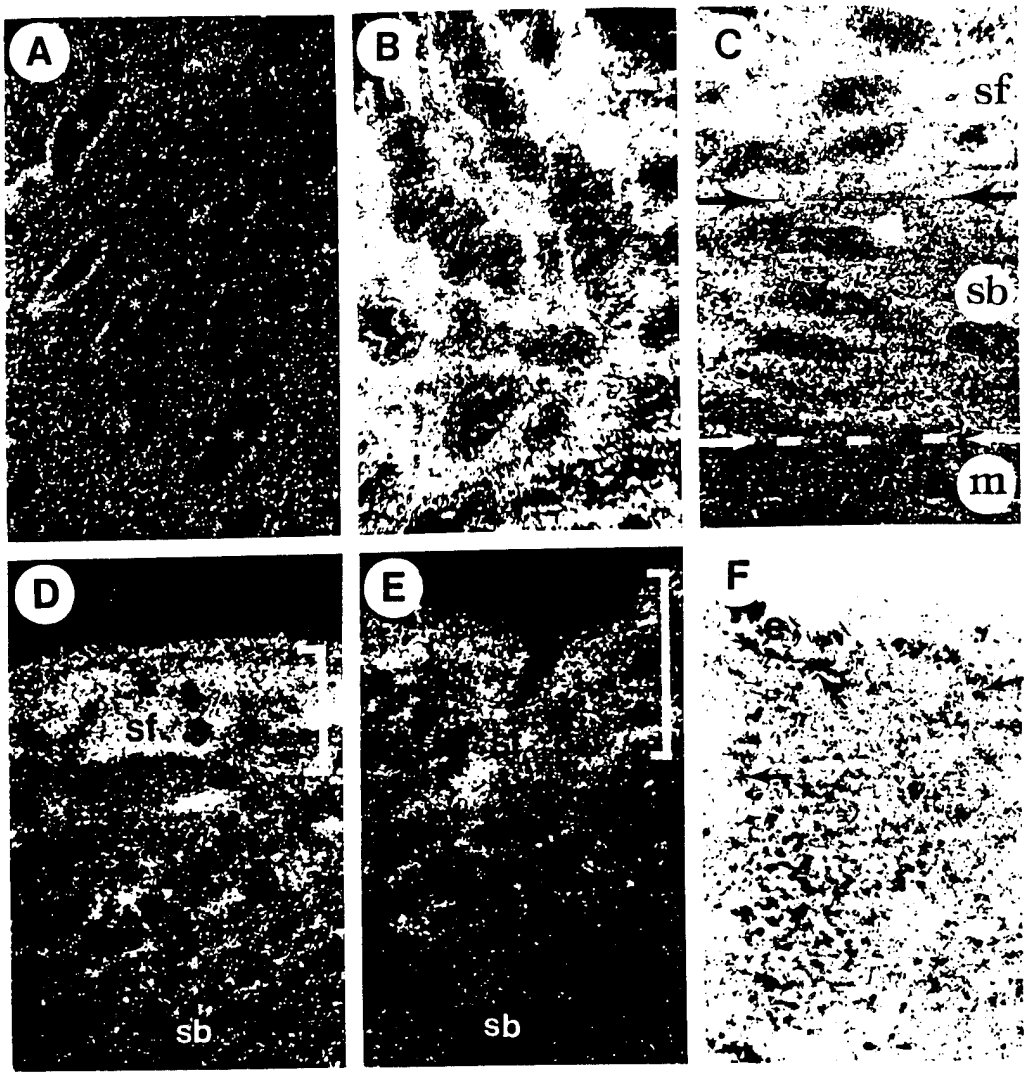
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Figure 3

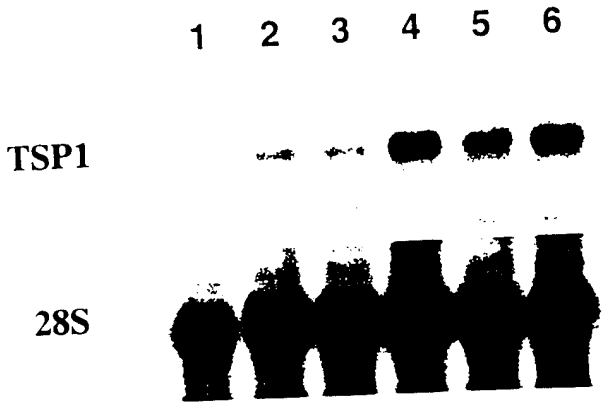
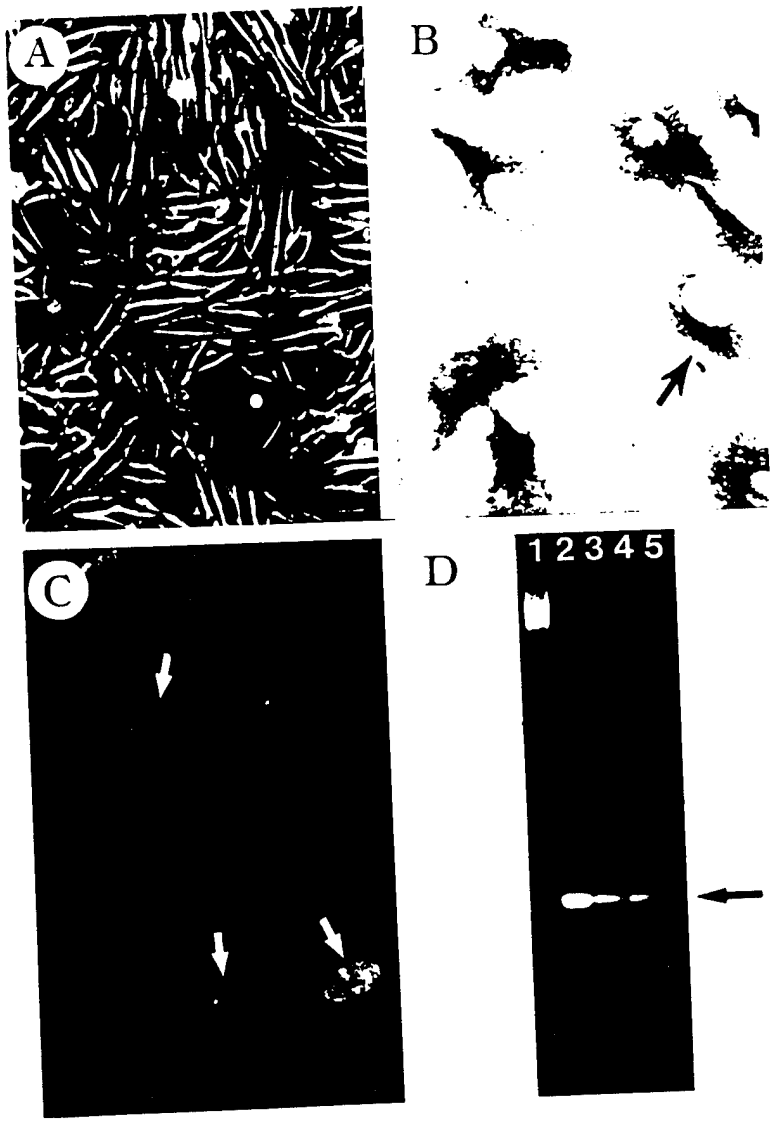


Figure 4



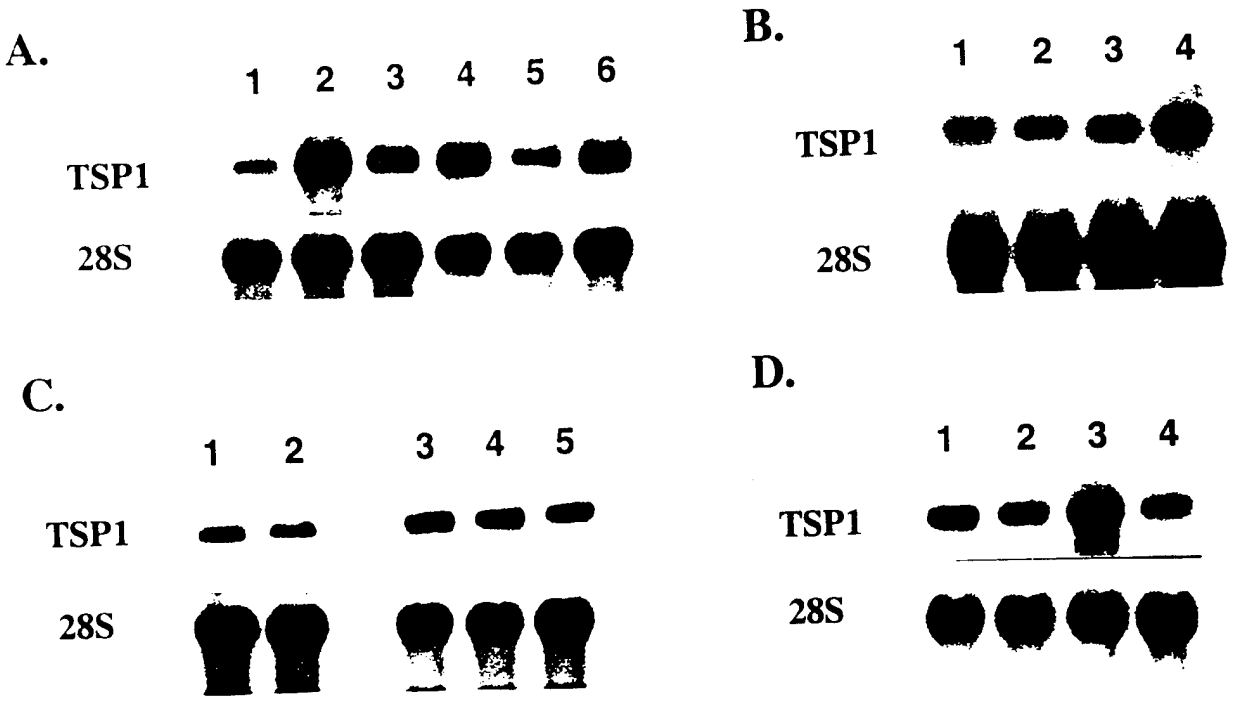
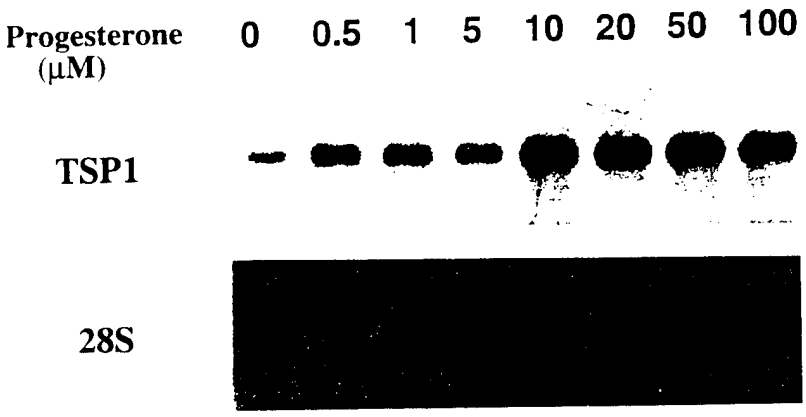

A.
1 2 3 4 5 6
TSP1
28S
B.
1 2 3 4
TSP1
28S
C.
1 2 3 4 5
TSP1
28S
D.
1 2 3 4
TSP1
28S

Figure 6

A.


Progesterone (μM)
0
0.5
1
5
10
20
50
100
TSP1
28S

B.

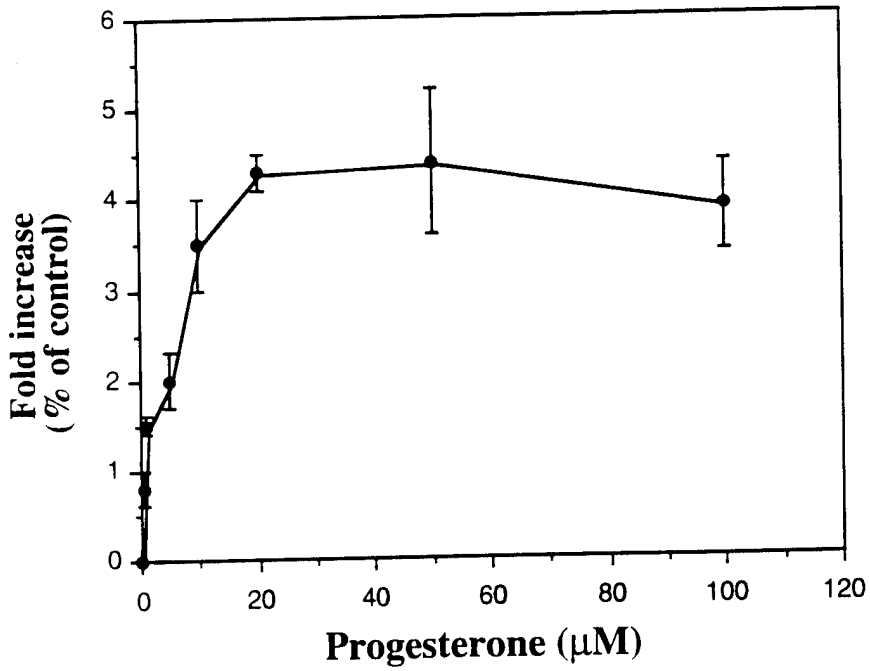
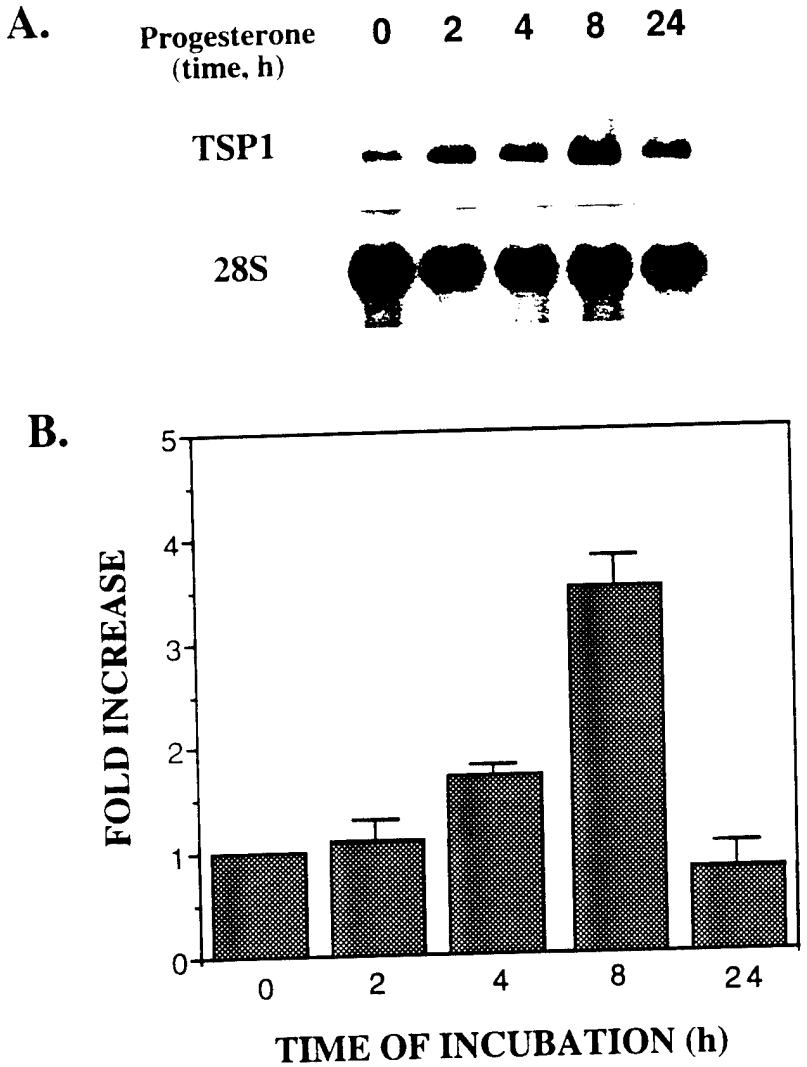

Fold increase (% of control)
0
1
2
3
4
5
6
0
20
40
60
80
100
120
Progesterone (μM)

Figure 7


A.
Progesterone (time, h)
0 2 4 8 24
TSP1
28S
B.
FOLD INCREASE
0
1
2
3
4
5
0 2 4 8 24
TIME OF INCUBATION (h)

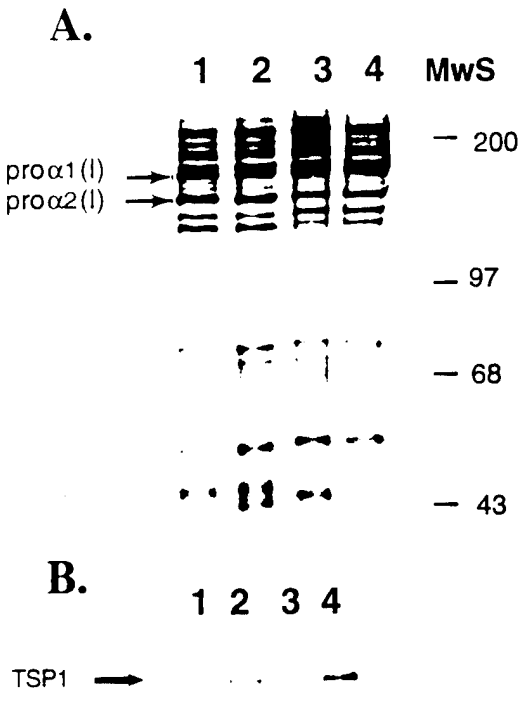
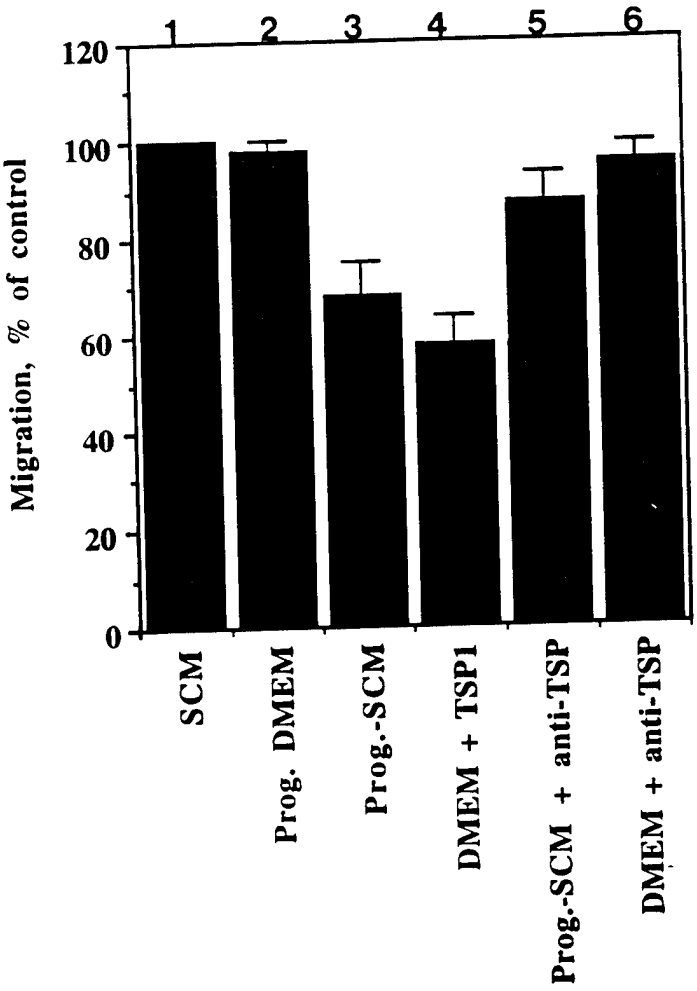

A.
1 2 3 4 MwS
proα1(I)
proα2(I)
200
97
68
43
B.
1 2 3 4
TSP1

Figure 9


1
2
3
4
5
6
120
100
80
60
40
20
0
Migration, % of control
SCM
Prog. DMEM
Prog.-SCM
DMEM + TSP1
Prog.-SCM + anti-TSP
DMEM + anti-TSP